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Lysine Post-Translational Modifications of Saccharomyces Cerevisiae Chromatin Proteins

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Abstract
DNA exists within the cell as part of a complex structure called chromatin which is comprised of many proteins, including histones, and participates in and influences every DNA-related process. Chromatin's proteins are modified post-translationally and this impacts their functions and in turn, the DNA processes in which they participate. However, the repertoire of post-translational modifications (PTMs), the enzymes that create and remove them, and their roles in chromosome biology are not fully understood. We have used the budding yeast Saccharomyces cerevisiae to investigate chromatin PTMs, specifically lysine modifications, through three avenues: the enzymes that regulate lysine PTMs, the histone chromatin lysine PTMs, and the non-histone chromatin lysine PTMs. First, we demonstrated that the JmjC domain-containing protein Kdm5 demethylates methylated lysine 4 of histone H3 in vitro and in vivo, refuting the longstanding hypothesis that lysine methylation is irreversible. Second, we confirmed and characterized monomethylation of lysine 20 of histone H4. Its abundance is highest at heterochromatic locations including rDNA, the silent mating type loci, and subtelomeres, lowest at euchromatic locations including centromeres and promoter/5' regions of genes, and intermediate inside genes. We observed a correlation between the locations of H4 K20A-mediated derepression and H4 K20me1 enrichment. Additionally, H4 K20me1 decreases globally during replicative ageing and may participate in survival during DNA damage. Our results refute the longstanding hypothesis that this PTM is not conserved in S. cerevisiae and potentially identify the first repressive budding yeast histone lysine methylation. Third, we investigated NuA4's lysine acetylation of the chromatin protein Spt16, identified by a previously published in vitro acetyltransferase screen. Mass spectrometry identified three acetylation sites in vivo and their substitution with unacetylatable arginines, but not the acetyllysine mimics glutamine, causes moderate heat-sensitivity. Our results suggest that Spt16 acetylation occurs in vivo and is necessary for heat-tolerance.

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Christopher R. Edwards

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ABSTRACT

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DNA exists within the cell as part of a complex structure called chromatin which is comprised of many proteins, including histones, and participates in and influences every DNA-related process. Chromatin's proteins are modified post-translationally and this impacts their functions and in turn, the DNA processes in which they participate. However, the repertoire of post-translational modifications (PTMs), the enzymes that create and remove them, and their roles in chromosome biology are not fully understood. We have used the budding yeast *Saccharomyces cerevisiae* to investigate chromatin PTMs, specifically lysine modifications, through three avenues: the enzymes that regulate lysine PTMs, the histone chromatin lysine PTMs, and the non-histone chromatin lysine PTMs. First, we demonstrated that the JmjC domain-containing protein Kdm5 demethylates methylated lysine 4 of histone H3 *in vitro* and *in vivo*, refuting the longstanding hypothesis that lysine methylation is irreversible. Second, we confirmed and characterized monomethylation of lysine 20 of histone H4. Its abundance is highest at heterochromatic locations including rDNA, the silent mating type loci, and subtelomeres, lowest at euchromatic locations including centromeres and promoter/5' regions of genes, and intermediate inside genes. We observed a correlation between the locations of H4 K20A-mediated derepression and H4 K20me1 enrichment. Additionally, H4 K20me1 decreases globally during replicative ageing and may

participate in survival during DNA damage. Our results refute the longstanding hypothesis that this PTM is not conserved in *S. cerevisiae* and potentially identify the first repressive budding yeast histone lysine methylation. Third, we investigated NuA4's lysine acetylation of the chromatin protein Spt16, identified by a previously published *in vitro* acetyltransferase screen. Mass spectrometry identified three acetylation sites *in vivo* and their substitution with unacetylatable arginines, but not the acetyllysine mimics glutamine, causes moderate heat-sensitivity. Our results suggest that Spt16 acetylation occurs *in vivo* and is necessary for heat-tolerance.

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Introduction

The nature of chromatin

DNA is a linear nucleic acid chain with regions, called genes, serving as templates for protein synthesis by the translation machinery. This is DNA's essential function and most processes occurring on DNA serve to facilitate or ensure the fidelity of this function. Transcription relays the protein-synthesis instructions to the translation machinery, DNA repair maintains the fidelity of these templates, DNA replication duplicates the DNA molecules to ensure that each daughter cell inherits a copy, and homologous recombination reorganizes the combination of gene versions that are present within each DNA molecule to promote genetic variability (Campbell, N.A., 1999).

DNA does not exist naked within the cell, but rather is part of a large and complex proteinacious structure called chromatin. Chromatin's components include the DNA, transcribed RNAs, and a variety of proteins participating in the above mentioned processes, but primarily proteins called histones. Histones exist in chromatin as octamers containing two copies each of the core histones H2A, H2B, H3, and H4. An additional linker histone, linker histone H1, exists in chromatin separate from the octamers. These octamers are integrated into chromatin as structures termed nucleosomes, each of which consists of one octamer with ~146bp of the linear DNA molecule wrapped around twice. Nucleosomes are distributed across the entire DNA molecule (with exceptions such as telomeric repeats) with variable length spaces in-between them along the DNA, resulting in a structure reminiscent of beads on a string. While "chromatin" can refer to the complex of DNA, RNA, histones, and non-histone

proteins, we will here use “chromatin” to also refer to DNA with histones (Luger, K., 1997; Campbell, N.A., 1999).

Each core histone has a globular domain that comprises the bulk of and mediates histone-histone interactions within the nucleosome, and an unstructured N-terminal tail that extends into the surrounding nucleoplasm. These core histones interact with one another via the “histone fold” within their globular domains and with the DNA strand that is wrapped around the octamer via numerous hydrogen bonds and positive charges derived from amino acid side chains on the nucleosome’s surface (Luger, K., 1997).

Each DNA molecule is partially condensed by being wrapped around many histone octamer thus allowing it to fit within the nucleus. Chromatin however can achieve additional levels of compaction. Adjacent nucleosomes and the linker histone H1 can interact to reduce the physical space in-between them, large stretches of chromatin may retract to form “30nm fibers,” and during mitosis, these higher-order chromatin structures achieve maximal compaction to form the famous chromosome structure (Li, G., 2011).

In addition to facilitating DNA compaction, nucleosomes can also stearically block access to DNA-interacting factors. *In vitro* transcription for example can occur on naked DNA but not so well on reconstituted chromatin, presumably since the nucleosomes block recruitment of the transcription initiation complex and translocation of the RNA polymerase (Pavri, R., 2006). Likewise, *in vivo* transcription involves nucleosome depletion at promoters and transcribed regions (Jiang, C., 2009), modulation of nucleosome structure ahead of RNA polymerase 2 (RNApol2) to allow its translocation, and restoration of nucleosome structure behind elongating RNApol2 to

prevent inappropriate intragenic transcription initiation (Belotserkovskaya, R., 2003; Fleming, A.B., 2008; Xin, H., 2009). Additionally, nucleosomes provide a surface with which chromatin-binding proteins can interact (discussed below) (Ruthenburg, A.J., 2007).

Nucleosomes thus participate in all chromatin-related processes and can be altered in three ways that influence these processes. First, nucleosomes can be incorporated into, evicted from, and translocated along chromatin, thereby altering what regions of DNA are accessible. These changes occur via histone chaperones that incorporate them (Burgess, R.J., 2010) and ATP-dependent histone remodelers that reposition or evict them (Saha, A., 2006). Nucleosomes' structures can also be modulated by FACT to change DNA accessibility in a way that is ATP-independent (Winkler, D.D., 2011). Second, the histone composition of nucleosomes can involve non-canonical (variant) histones. For example, Htz1 can replace H2A and is associated with promoters, H2AX can replace H2A and participates in DNA damage repair by being phosphorylated, MacroH2A can replace H2A in the inactive X Barr body, and Centromeric Protein A (CENPA) can replace H3 at centromeres and participates in centromere and kinetochore function (Talbert, P.B., 2010). Third, histones can be modified post-translationally (below).

The nature of post-translational modifications

Post-translational modifications (PTMs) are any change to a protein's structure that occurs after translation and includes proteolyses, conformational changes, and the covalent attachment or removal of chemical groups, usually to amino acid side chains. The effect is a diversification of the protein's local structure, potentially affecting protein

interactions or the protein itself, and this provides several advantages to the cell. First, proteins can be synthesized in an inactive form that only becomes active after PTM. For example, some Caspases cannot participate in the apoptotic program until they have been activated by cleavage (Pop, C., 2009). Second, chemical alterations to amino acid side chains limit the number of tRNAs that must exist. Without PTMs, codons and tRNAs would have to exist for acetyllysines, methyllysines, phosphoserines, etc. Third, it is more economical to modify a protein than to replace it. For example, if a protein within a complex has to be acetylated, it is more energy-efficient and less disruptive to the complex if the acetyl group is added post-translationaly than if the protein is removed and replaced with an acetyl protein. Fourth, it is faster to alter a protein by PTM than to synthesize a new one.

Many PTMs exist. Isopeptide bonds can be hydrolyzed and caspases are an example (Pop, C., 2009). Prolyl residues can be isomerized between *cis* and *trans* conformations (Lu, K.P., 2007). Fpr4 for example can isomerizes proline 38 of histone H3, thereby affecting lysine 36 methylation that participates in transcription (Nelson, C.J., 2006). Small proteins can be fused to lysines and include Ubiquitin and related proteins like SUMO (Small Ubiquitin-related Modifier) and Nedd8 (Kerscher, O., 2006). Most proteins can be ubiquitylated and many such as p53 can be SUMOylated and neddylated. Small molecules can also be covalently bound to amino acid side chains and this is a very diverse group of PTMs. Phosphate groups can be fused to the hydroxyl oxygens of serines, threonines, and tyrosines (Ubersax, J.A., 2007). Proline's cyclical backbone can be hydroxylated (Gorres, K.L., 2010). Chains of ADP-ribose can be covalently bound to glutamic or aspartic acid (Schreiber, V., 2006). Methyl groups can be covalently attached to the guanidinium nitrogens of arginines (Di Lorenzo, A.,

2011). Finally, acetyl, butyryl, propionyl, crotonyl, and methyl groups can be fused to the epsilon amine nitrogen of lysines (Yang, X.J., 2008).

Each PTM has advantages and disadvantages for the cell. Isopeptide bond hydrolyzation is the most dramatic structural change but is irreversible. The addition of small proteins provides a relatively large structure that could have strong stearic effects and provides the opportunity for chain formation since the attached proteins could also be modified. Ubiquitin for example can form polyubiquitin chains that have different functions depending on the chains' structure and monoubiquitination of histones is proposed to act as a wedge to force nucleosomes apart (Kerscher, O., 2006). Phosphate attachment is a relatively small structural change but creates a negative charge. Acetyl attachments to lysine are also a relatively small structural change but remove a positive charge and add a carbonyl carbon to the end of the side chain (Chen, Y., 2007; Zhang, K., 2009; Tan, M., 2011). Adjacent acetylations can also sometimes work synergistically, allowing for a graded effect that depends on the abundance of acetylations (Yang, X.J., 2008). Finally, methylation of arginines and lysines causes a relatively small structural change but leaves the positive charge present. Importantly, methylation allows for a high degree of structural diversification at a single residue. Whereas the other small molecule modifications are either present or absent, arginines can be un-, mono-, asymmetrically di-, or symmetrically dimethylated, and lysines can be un-, mono-, di-, or trimethylated. In-general, we will use "post-translational modification" to refer to the covalent attachment of molecules.

The mechanisms by which post-translational modifications function

PTMs can generally affect their target proteins through three mechanisms. First, PTMs can directly alter the modified protein's structure or function. For example, the spontaneous compaction of *in vitro* reconstituted chromatin can be inhibited by acetylated histone H4 lysine 16 (H4 K16ac) and ubiquitylated H2B (Shogren-Knaak, M., 2006; Lu, X., 2008; Fierz, B., 2011). The mammalian gluconeogenesis enzyme Pck1 is acetylated and this promotes its enzymatic activity *in vitro* (Lin, Y.Y., 2009). Additionally, some Caspases can autoproteolyze to activate their apoptotic ability (Pop, C., 2009).

Second, PTMs can interact with proteins to affect their function without affecting their recruitment. For example, H2B ubiquitylation is necessary for H3 K79 methylation by Dot1 and hDot1L *in vivo*. On *in vitro* reconstituted chromatin however, H2B ubiquitylation promotes hDot1L's H3 K79 histone methyltransferase (HMT) activity without affecting its recruitment, implying an allosteric affect that promotes enzymatic activity (McGinty, R.K., 2008).

Third, PTMs can promote or block the recruitment of other molecules. For example, the side chain amine group hydrogen of H3 K56 can hydrogen bond with DNA at the nucleosome's DNA entry-exit site. Acetylation of this residue removes this H-bond resulting in decreased nucleosome-DNA contact and increased accessibility of nucleosomal DNA *in vitro* (Masumoto, H., 2005). The greatest effect of PTMs on protein interactions however is on the recruitment of "readers," proteins that bind other modified proteins dependent on their modifications. A variety of domains mediate these interactions by "reading" the structural changes caused by the PTMs. For example, SH2 and BCRT domains bind phosphopeptides, bromodomains bind acetyllysines, ubiquitin-associated domains bind polyubiquitins, and PH fingers and chromo, tudor, and MBT

domains bind methyllysines (Yun, M., 2011). This may be PTMs' most important function and as such, a large repertoire of known interactions exists.

For example, the histone methyltransferase MMSET is recruited to MDC1 at sites of DNA damage via interactions between MMSET's phosphorylated serine 102 and MDC1's phospho-binding BRCT domain (Pei, H., 2011). Monoubiquitylated K123 of *S. cerevisiae* histone H2B recruits the COMPASS complex subunit Cps35 to promote H3 K4me3 *in vivo* (Lee, J.S., 2007) and simultaneously blocks recruitment of the HMT Set2 to prevent H3 K36 methylation *in vivo* and *in vitro* (Wyce, A., 2007). Interactions between K48-linked polyubiquitin chains and ubiquitin-associated domains recruits polyubiquitylated proteins to the proteasome for degradation (Welchman, R.L., 2005). The budding yeast SAGA histone acetyltransferase (HAT) complex is recruited to the promoter and 5' regions of genes via interactions with H3 K4me3 (Bian, C., 2011). Sir3 of the Sir2/3/4 complex binds chromatin via interactions with the H4 K16 and H3 K79 epitopes and is blocked by H4 K16ac and H3 K79me (Altaf, M., 2007). *Shizosaccharomyces pombe* Crb2 binds H4 K20me2 and its mammalian counterpart 53BP1 binds H4 K20me2 and p53 K370me2 via tandem tudor domains during DNA damage (Botuyan, M.V., 2006; Huang, J., 2007). Trimethylated H3 K9 of mammalian heterochromatin can recruit HP1 to promote proper chromatin structure (Schotta, G., 2004). H4 K20me1 recruits L3MBTL1 via an MBT domain to promote chromatin compaction *in vitro* (Trojer, P., 2007). *S. cerevisiae* intragenic H3 K36me3 recruits the small Rpd3 complex which then deacetylates histones to promote sufficient chromatin compaction levels (Carrozza, M.J., 2005). Finally, H3 K27me3 participates in Polycomb complex recruitment to promote transcription repression (Simon, J.A., 2009).

The biological significance of post-translational modifications

PTMs are most well-studied on histones and through these three mechanisms play important roles in almost every chromatin-related process. Almost every known modification type participates in DNA damage repair, occurring on both histones and the repair machinery to coordinate the many protein interactions (Lukas, J., 2011). H4 S1 phosphorylation during *S. cerevisiae* meiosis promotes chromatin compaction (Krishnamoorthy, T., 2006). Chromatin compaction is directly influenced by H2B ubiquitylation and H4 K16ac (Shogren-Knaak, M., 2006; Fierz, B., 2011), and by the recruitment of chromatin compaction proteins like L3MBTL1 by H4 K20me1 (Trojer, P., 2007) and heterochromatin proteins like HP1 by H3 K9me3 (Schotta, G., 2004). H3 K56ac and H4 K20me1 respectively participate in the DNA damage response and DNA replication (Masumoto, H., 2005; Brustel, J., 2011). Finally, transcription is orchestrated by numerous PTMs; histone acetylation occurs at promoter and 5' regions to make DNA accessible for transcription initiation, histone deacetylation occurs at middle and 3' regions to make DNA inaccessible for transcription initiation, H3 K4me3 occurs at 5' regions, H3 K36me3 occurs at middle and 3' regions to recruit the deacetylases, H3 K79 methylation occurs intragenically perhaps to exclude the Sir proteins, H2B ubiquitylation occurs intragenically to coordinate these three methyllysines, H2A ubiquitylation occurs to repress transcription with the Polycomb complexes, and H3 K9 and H4 K20 monomethylation may participate in a repressive manner as well, although how is unclear.

It is increasingly clear that PTMs are not limited to histones but rather occur on and regulate many proteins in the cell. Histones are thus a useful model for understanding the biochemistry and role of PTMs on non-histone proteins and,

considering the extent to which histone are modified, are a particularly useful model for understanding the coordination and crosstalk that could occur on heavily decorated non-histone proteins like p53. While our current understanding of non-histone PTMs is in its infancy, there are already many examples of their importance. Phosphorylations occurs on many DNA damage repair proteins and are critical mediators of intracellular signaling pathways (Lukas, J., 2011). Acetylation occurs on Hsp90 to influence its protein chaperone functions (Kovac, J.J., 2005), p53 to influence its transcription activation activities (Bode, A.M., 2004), Pck1 to influence its gluconeogenesis enzymatic activity (Lin, Y.Y., 2009), and many other proteins including alpha tubulin, GDH, the hormone receptor LXR, Foxo1 and eNOS, beta-catenin, PGC-1alpha, CPS1, and Ku70 (Close, P., 2010; Yang, X.J., 2008). Methylation occurs on p53 to affect its gene-association and transcriptional activity, TAF10 to affect its transcriptional activity, the chloroplast protein Rubisco, the mitochondrial protein Cytochrome C, the ribosomal proteins Rpl23ab and Rpl12, and the receptor tyrosine kinase VEGFR1 (Huang, J., 2008).

Considering the range of biological roles with which PTMs are associated, it is unsurprising that they are increasingly connected to diseases. Histone deacetylase (HDAC) inhibitors are noted for their anti-tumor properties (Bode, A.M., 2004). SIRT6 was recently shown to prevent the metabolic shift of glucose away from cellular respiration outside of hypoxic conditions (Zhong, L., 2010). This inappropriate shift towards anaerobic glycolysis is reminiscent of the “Warburg” effect that occurs in tumor cells. Translocations involving the mammalian H3 K4 methyltransferase MLL are linked to Acute Myeloid Leukemia and knockdown and pharmacological inhibition of the acetyllysine-binding protein Brd4 recently demonstrated potent toxicity to these cancer

cells (Zuber, J., 2011). Finally, increased H4 K16ac during *S. cerevisiae* replicative ageing limits yeast replicative lifespan (Dang, W., 2009).

Enzymes that create and remove post-translational modifications

The covalent attachment and removal of these proteins and small molecules are facilitated by many enzymes. Ubiquitin and related proteins are attached to target lysines by a series of three enzymes (E1-3). Ubiquitin's C-terminus is first covalently attached to an E1 ubiquitin-activating enzyme in an ATP-dependent manner, then transferred to an E2 ubiquitin-conjugating enzyme, then transferred to a target protein by an E3 ubiquitin-ligase (Kerscher, O., 2006). The result is a peptide bond between a lysine epsilon nitrogen and the ubiquitin C-terminal carboxylic acid that can be hydrolyzed by ubiquitin proteases to create a free ubiquitin and restored lysine. Phosphates are transferred from ATP to the hydroxyl oxygens of serine, threonine, and tyrosine side chains by kinases resulting in a negative charge for the amino acid. A phosphatase (phosphorylase) can then hydrolyze to remove the phosphate group (Ubersax, J.A., 2007).

Acetyl (and presumably propionyl, butyryl, and crotonyl) groups are covalently attached to the epsilon nitrogen of lysine side chains by acetyltransferases. This process is proposed to involve deprotonation of the amine group's hydrogen, resulting in the nitrogen performing a nucleophilic attack on the carbonyl carbon of an acetyl coenzyme-A molecule. The result is an acetyl group covalently bound to the epsilon nitrogen by its carbonyl carbon, increasing the lysine side chain's size, adding a polar carbonyl group, and reducing its net charge to neutral (Berndsen, C.E., 2008). Two classes of deacetylases remove these acetyl groups, one of which requires the cofactor

NAD+. Up to three methyl groups can be covalently attached to lysine, resulting in relatively small or large structural changes and maintenance of the positive charge. This reaction is facilitated by methyltransferases that may deprotonate the lysine's epsilon nitrogen, after which the nitrogen attacks the methyl group of an S-adenosyl methionine. The methyl group then transfers to the lysine leaving behind an S-adenosyl homocysteine (Xiao, B., 2003).

While most known chemical modifications have enzymes to facilitate their removal, lysine methylations were long considered permanent due to the absence of any identified lysine demethylase. The closest enzyme to a demethylase was PADI4, an arginine "demethylase" that actually changes methylarginine into citrulline rather than unmethylated arginine (Thompson, P.R., 2006). The reversibility of methylation was demonstrated however when *in vitro* lysine demethylase activity was shown for LSD1 (BHC110) via its amine oxidase domain (Shi, Y., 2004) and JHDM1 via its JmjC domain (Tsukada, Y., 2005). Both domains function by hydroxylating the target methyl group making a carbanolamine that then "falls off" as formaldehyde. The epsilon nitrogen binds a proton in its place resulting in the lysine having one less methyl group. LSD1 achieves this hydroxylation by removing a hydrogen from both the target methyl group and epsilon nitrogen, resulting in an imine (-N=C-) that is hydrated to hydroxylate the methyl group. This mechanism cannot target trimethyllysines since their nitrogen has no hydrogen to remove. JmjC domains however bypass this restriction by directly hydroxylating the target methyl group. The enzyme forms a complex with iron, alpha-ketoglutarate, and oxygen, breaking alpha-ketoglutarate into succinate and carbon dioxide and producing an oxoferryl species that hydroxylates the methyllysine (Shi, Y., 2007).

Methods to study post-translational modifications

PTM studies generally involve observing when and where in the cell (and along the genome for a chromatin PTM) it is present. While mass spectrometry can be used in some cases, antibodies specific to the modification and surrounding epitope are often the preferred method. Peptide competitions can argue that these antibodies recognize their intended targets, but only abrogation of the modification can truly rule out cross-reactivity with other epitopes *in vivo*. While loss or decrease of the PTM can be achieved by overexpressing, knocking down, or knocking out the responsible enzyme, these approaches have three disadvantages. First, enzyme redundancy might minimize changes to the modification's levels. Second, if the enzyme modifies multiple residues on the same protein, as sometimes occurs with histone acetylation, ruling out cross-recognition by the antibody may be difficult. Third, this experiment is impossible if the responsible enzyme is unknown.

Another method to demonstrate antibody specificity is to mutate the modified residue, preferably such as to mimic the unmodified state. Unlike enzyme perturbations, this guarantees that the PTM-of-interest is eliminated, better ensures that similar marks on the same protein are not affected, and does not require the responsible enzyme to be known. This method however has the disadvantage of eliminating all marks on that residue, meaning it cannot rule out that the antibody recognizes other modifications of that amino acid.

PTM studies also involve decreasing or increasing its levels to determine what it does. While this can be accomplished by enzyme overexpression, knockdown, or knockout, this has several disadvantages. First, enzyme redundancy might minimize changes to the modification's levels. Second, if the enzyme targets multiple proteins, all

of these PTMs may be affected. Third, the responsible enzyme must be known. Mutating the modified residue is again a better alternative for several reasons. Unlike enzyme perturbations, this guarantees that the PTM-of-interest is eliminated, better ensures that other marks created by the enzyme are not affected, and does not require the responsible enzyme to be known. Further, some substitutions can mimic the constitutive presence of PTMs. Aspartic acid for example can be a phospho mimic while glutamine can be an acetyllysine mimic. Therefore, while residue substitutions will eliminate all of a residue's modifications, PTM mimics can sometimes reveal which residue's modifications perform which function.

For these reasons, amino acid substitution experiments are critical for PTM studies and add much when paired with enzyme perturbation experiments. Amino acid substitutions can be difficult in higher eukaryotes however. To reveal a PTM's function, the modified protein's endogenous genes must be removed and replaced in the genome, or on a plasmid, by a gene containing the amino acid substitution. Such targeted knockouts are difficult in some higher eukaryotes and if the gene to be deleted has multiple copies, as is the case for histone genes, then a substitution experiment may be unfeasible even in genetically tractable higher eukaryotes like mice. An alternative approach is to knock down the endogenous mRNA and simultaneously express a gene that is knockdown-resistant and contains the residue substitution. This method however is only as effective as the RNAi.

Yeast are a useful alternative model organism to use for PTM studies, especially for chromatin. Like mice, they are genetically tractable. Unlike mice however, they possess fewer histone genes, making histone residue substitution experiments relatively easy. Since they are unicellular, cell-cycle and molecular events can be studied without

resorting to the *in vitro* conditions of mouse cell cultures or having to tease apart tissue-specific effects. Conversely, yeast do not offer the chance to study the effects of PTMs on cell fate, development, or tissue-specific functions. Importantly, as described below, *S. cerevisiae* have chromatin that lacks several well-studied PTMs and components that are found in higher eukaryotes. Due to this "simplicity," yeast may make it easier to tease apart the molecular properties of chromatin and protein PTMs, but cannot guide the research of all the higher eukaryote chromatin components.

Saccharomyces cerevisiae chromatin post-translational modifications

S. cerevisiae possess all the major types of modifications including lysine acetylation, ubiquitylation, sumoylation, and methylation, arginine methylation, and serine, threonine, and tyrosine phosphorylation. Many well-known histone PTMs are conserved including the H3 and H4 tail acetylations, H4 K16ac, H2B monoubituitylation, and all three methylation states of H3 K4, H3 K36, and H3 K79. Methylation at these three lysines are greatly conserved in eukaryotes, with genome-wide patterns having many similarities between budding yeast and mammals. Many PTM enzymes are also conserved including the H3 K4 HMT COMPASS complex (the MLL complex in mammals) and the H3 K79 HMT Dot1 (hDot1L in humans) (Martin, C., 2005).

Notably, several well-studied PTMs and enzymes in higher eukaryotes lack corresponding PTMs and orthologs in *S. cerevisiae*. Methylated H3 K9, H3 K27, and H4 K20 have not been identified, although the lysines are conserved. H2A ubiquitylation has also not been found. Obvious orthologs of the corresponding mammalian HMTs and ubiquitin ligases are also not present. It is intriguing that the major histone modifications considered absent in budding yeast (listed above) are generally linked to

transcriptional repression or chromatin compaction and further, *S. cerevisiae* are not known to have verified repressive histone lysine methylations. *S. cerevisiae* chromatin is thus considered to be less compacted and regulated by a smaller number of histone PTMs than higher eukaryote chromatin (Sautel, C.F., 2007).

Important questions about chromatin post-translational modifications

Chromatin PTM research can be organized into three avenues: investigating the PTM enzymes, investigating the histone chromatin PTMs, and investigating the non-histone chromatin PTMs. Numerous important questions remain to be answered within each field. Enzymes, for example, are unknown for the recently identified *S. cerevisiae* histone PTMs H2B K37 (Gardner, K.E., 2011) and H3 K42 (Hyland, E.M., 2011). Histone methyllysine demethylases were also only recently discovered and many are predicted but poorly characterized in every model organism (Klose, R.J., 2006).

Histone chromatin PTMs are not fully understood in that their full repertoire is not known and many that are known are not sufficiently characterized. Mass spectrometry of mammalian histones for example recently identified 67 new marks including two new modification types, lysine crotonylation and tyrosine hydroxylation (Tan, M., 2011), and another mass spectrometry approach a few years ago also reported new modifications in multiple organisms including *S. cerevisiae* (Garcia, B., 2007). Since these approaches identified so many previously unknown PTMs but failed to identify other known PTMs, many marks may have been missed and await discovery. Other histone marks such as H4 K20 methylation are already known and well-studied, but considered rather complicated due to being linked to a large number of processes and due to occasionally conflicting reports (Brustel, J., 2011; Yang, H., 2009).

Similarly, non-histone chromatin PTMs are not fully understood in that the repertoire of known PTMs is still in its infancy and many remain to be identified and characterized. p53 for example is subject to almost every PTM type and illustrates how extensive their regulation of non-histone chromatin-associated proteins can be (Bode, A.M., 2004). Methylation and acetylation are increasingly reported on non-histone proteins as well (Huang, J., 2008; Close, P., 2010). Importantly, a recent report identified 91 *in vitro* targets of the *S. cerevisiae* HAT NuA4, including many chromatin-associated proteins, and they await individual confirmation and characterization (Lin, Y.Y., 2009).

Our studies of Saccharomyces cerevisiae chromatin post-translational modifications

We have chosen to investigate chromatin PTMs through the three previously described avenues of research: the PTM enzymes, the histone chromatin PTMs, and the non-histone chromatin PTMs. We have chosen to use the budding yeast *S. cerevisiae* as our model organism since it provides technological advantages including the critical ability to generate amino acid substitutions in histones and other proteins. We have chosen to focus on PTMs of lysine since they are the most varied. Finally, we have also chosen to focus primarily, though not solely, on lysine methylation since this is lysine's most complex and functionally diverse modification and since its recently discovered reversibility makes it an important area of investigation. Below are the three specific aims which we intended to carry out in order to better understand lysine chromatin PTMs in *S. cerevisiae*.

1) Histone Lysine Demethylation in *S. cerevisiae*:

Lysine methylation, long considered permanent, was shown to be reversible by the mammalian LSD1 amine oxidase domain (Shi, Y., 2004) and the JHDM1 JmjC domain (Tsukada, Y., 2005) a few years ago. Since JmjC domains are predicted in almost every model organism and five *S. cerevisiae* proteins (Klose, R.J., 2006), important questions in this avenue of chromatin PTM research are which predicted JmjC domain proteins are demethylases, which chromatin methyllysines they target, and what biological processes they influence. We have chosen to ask whether the *JHD2* protein product Kdm5 (aliases Jhd2, Yj89, and Yjr119c) demethylates histone methyllysines *in vitro* and *in vivo* and whether it regulates methyllysines during transcription.

2) Histone Lysine Methylation is *S. cerevisiae*:

We have chosen to investigate histone methyllysines in *S. cerevisiae* by attempting to confirm the presence of and characterize H4 K20me1. H4 K20 is mono-, di-, and trimethylated in many higher eukaryotes. The monomethylated state is especially interesting since it seems to be distinct from the other states in terms of the responsible enzymes, genomic enrichment, reader proteins, and biological functions. However, this PTM is considered complicated, partly due to occasionally conflicting reports, but mainly due to the large number of functions to which it is linked, although it is generally considered associated with transcriptional repression and chromatin compaction. Briefly, mammalian H4 K20me1 is considered associated with repressed regions, is enriched inside genes relative to flanking regions, promotes transcription repression, recruits chromatin compactions proteins, promotes compaction *in vitro*, may promote origin licensing, is cell cycle-regulated, and may participate in DNA replication, mitosis,

and DNA damage repair (Brustel, J., 2011; Yang, H., 2009). Further, studies involving perturbing its methyltransferase Set8 (Pr-Set7) are complicated by the fact that this may alter the di- and trimethylated states and the fact that Set8 targets p53 and perhaps other proteins (Shi, X., 2007). The genetically tractable *Schizosaccharomyces pombe* also possess H4 K20me1, but using it as a model to clarify H4 K20me1's role is limited since H4 K20me2, 3 are also present, the same enzyme may create all three states, and functional differences between the states are unclear (Sanders, S.L., 2004).

Unlike most higher eukaryotes, *S. cerevisiae* was considered to lack methylation of H4 K20, as well as H3 K9 and H3 K27. These three lysines are conserved from budding yeast to humans and their methylation in higher eukaryotes is associated with transcriptional repression or chromatin compaction. In contrast, lysines H3 K4, H3 K36, and H3 K79 and their methylation are conserved from budding yeast to humans and this methylation is associated with transcriptional activity or an open chromatin state (Martin, C., 2005). *S. cerevisiae* thus possess all six conserved lysines and the three "active" methylations but lack the three "repressive" methylations. As such, budding yeast are considered to have chromatin that is generally more "open" than higher eukaryote chromatin.

Since a mass spectrometry report suggested that monomethylated H4 K20 exists in *S. cerevisiae* (Garcia, B.A., 2007), we chose to confirm and characterize this mark for several reasons. First, since budding yeast are excellent for studying histone PTMs, and since the di- and trimethylated states may be absent, unlike in *S. pombe*, this would be an excellent opportunity to study the role of this particular methylation state of this lysine. Second, if this mark were associated with repression, this would refute the longstanding notion that repressive *S. cerevisiae* histone methyllysines do not exist.

3) NuA4 Acetylation of Spt16 in *S. cerevisiae*:

We have chosen to investigate lysine modifications of non-histone chromatin proteins by attempting to confirm and characterize the acetylation of Spt16. Using a budding yeast proteome microarray, the HAT NuA4 was recently shown to *in vitro* acetylate 91 proteins, many of which are chromatin-related (Lin, Y.Y., 2009). One *in vitro* target was Spt16, part of the yFACT complex that is essential and participates in transcription elongation and possibly DNA replication and repair. Since this would be an opportunity to expand the repertoire of non-histone acetylation targets *in vivo*, shed light on NuA4's function, and elucidate the regulation of a critical chromatin protein, we chose to confirm and characterize the acetylation of *S. cerevisiae* Spt16.

DISSERTATION CHAPTER 2

Histone Lysine Demethylation in *Saccharomyces cerevisiae*

Chapter Outline

Section 1 Chapter Summary & Explanation of My Contributions

We sought to characterize the *Saccharomyces cerevisiae* JmjC domain-containing protein Kdm5 (*JHD2*) *in vivo* and *in vitro*. The work resulted in the following publication:

Ingvarsdottir, K., Edwards, C., Lee, M.G., Lee, J.S., Schultz, D.C., Shilatifard, A., Shiekhattar, R., and Berger, S.L. Histone H3 K4 Demethylation During Activation and Attenuation of *GAL1* Transcription in *Saccharomyces cerevisiae*. Molecular & Cellular Biology. Volume 27, 2007, 7856-7864.

This work and its supplementary materials are respectively presented in sections two and three of this chapter. Kristin Ingvarsdottir, a graduate student in the lab, was first author and performed the *in vivo* characterizations. My contributions were the *in vitro* characterizations (Figure 1) and a protein alignment of Kdm5 and a mammalian homolog called Jarid1d (Supp. Figure 1) and resulted in a second authorship. Section four contains an *in vivo* experiment and additional discussion points that are not present in the article and were performed and written by me.

To perform the *in vitro* characterization, I cloned *JHD2* into pFastBac for inset cell expression, purified FLAG-Kdm5, and used it in *in vitro* demethylation reactions with core histones or histone H3 tail peptides. Demethylation was observed via western analyses with methyl-specific antibodies. Kdm5 decreased H3 K4me2, 3 levels on core histones, and very slightly decreased H3 K4me1 levels. Kdm5 also targeted H3 peptides with mono-, di-, or trimethylated K4, demonstrating specificity for all three methylation states. While H3 K4me1 peptides were demethylated, reactions with H3 K4me2, 3 peptides resulted in an accumulation of H3 K4me1, 2, consistent with a distributive mechanism in which it removes methyl groups one at a time, creating lower methyl states as intermediates (Frederiks, F., 2008). Substitution of a predicted catalytic residue abrogated demethylase activity. Kdm5 also did not demonstrate activity on

methylated H3 K4 of nucleosomes (data not shown), similar to its mammalian ortholog Jarid1d. The additional unpublished *in vivo* experiment consisted of overexpressing *JHD2 in viv*o and observing that this produces a global decrease in H3 K4me3 levels.

Section 2 Kdm5 Demethylates Methylated H3 K4 in *Saccharomyces cerevisiae*

ABSTRACT

In mammalian cells, histone lysine demethylation is carried out by two classes of enzymes, the LSD1/BHC110 class and the jumonji class. The enzymes of the jumonji class in the yeast *Saccharomyces cerevisiae* have recently also been shown to have lysine demethylation activity. Here we report that the protein encoded by YJR119c (termed KDM5), coding for one of five predicted jumonji domain proteins in yeast, specifically demethylates trimethylated histone H3 lysine 4 (H3K4me3), H3K4me2, and H3K4me1 in vitro. We found that loss of *KDM5* increased mono-, di-, and trimethylation of lysine 4 during activation of the *GAL1* gene. Interestingly, cells deleted of *KDM5* also displayed a delayed reduction of K4me3 upon reestablishment of *GAL1* repression. These results indicate that K4 demethylation has two roles at *GAL1*, first to establish appropriate levels of K4 methylation during gene activation and second to remove K4 trimethylation during the attenuation phase of transcription. Thus, analysis of lysine demethylation in yeast provides new insight into the physiological roles of jumonji demethylase enzymes.

INTRODUCTION

Histone posttranslational modifications are centrally involved in genome regulation. Histone methylation occurs at both lysine (K) and arginine residues and plays a key role in chromatin organization and transcriptional regulation (Kouzarides, T., 2002; Margueron, R., 2005; Martin, C., 2005). In particular, methylation at lysine residues of histone H3 and H4 during gene regulation has been characterized extensively and linked to either gene activation (e.g., H3K4, H3K36, and H3K79) or repression (e.g., H3K9, H3K27, and H4K20). Histone H3 lysine methylation in the yeast *Saccharomyces cerevisiae* has been reported to occur at three residues (K4, K36, and K79) (Martin, C., 2005; Sims, R.J., 2003), and each correlates with gene activation. The methylation reaction is catalyzed either by many lysine-specific methyltransferases within the large SET domain family (all known sites except H3K79) or by the unrelated Dot1 (H3K79) (Shilatifard, A., 2006).

Until recently, histone lysine methylation was speculated to be irreversible and therefore possibly a true epigenetic modification persisting through cell division. However, two families of demethylation enzymes capable of targeting methylated lysine residues have now been identified (Cloos, P.A., 2006; Cuthbert, G.L, 2004; Fodor, B., 2006; Klose, R.J., 2006; Lee, M.G., 2005; Shi, Y., 2004; Tsukada, Y., 2006; Wang, Y., 2004; Whetstine, J.R., 2006; Yamane, K., 2006). LSD1, a catalytic engine of multicomponent corepressor complexes, was the first demonstrated lysine demethylase, reversing dimethylated histone H3 lysine 4 (H3K4me2) (Lee, M.G., 2005; Shi, Y., 2004; Shi, Y.J., 2005). However, this enzyme is unable to remove methyl groups from trimethylated lysine because of the inherent limitation of its enzymatic reaction mechanism. In contrast, a second class of histone lysine demethylases, consisting of the

JmjC domain containing proteins, are able to demethylate not only di-but also trimethylated histone marks (Cloos, PA., 2006; Fodor, B.D., 2006; Klose, R.J., 2006; Tsukada, Y., 2006; Yamane, K., 2006). Only recently have demethylases targeting the trimethylated form of H3K4 been characterized. The four members of the JARID family in mammalian cells contain a JmjC domain which is critical for their H3K4 demethylation activity (Christensen, J., 2007; Iwase, S., 2007; Klose, R.J., 2007; Lee, M.G., 2007). These enzymes have been shown to have in vivo effects on H3K4 methylation status, both on a global scale and at specific genes where they also affect the transcription levels. Studies of H3K4 demethylases in lower organisms are not as far advanced as those of mammalian counterparts. Lid, the only homolog of the JARID family in *Drosophila melanogaster*, has been shown to have H3K4 demethylation activity in vivo on a global scale (Eissenberg, J.C., 2007; Lee, N., 2007; Secombe, J., 2007). The same is true for JMJ2, the fission yeast homolog (Huarte, M., 2007), and YJR119c, the budding yeast homolog (Liang, G., 2007; Seward, D.J., 2007; Tu, S., 2007) of the JARID family. However, it remains to be established whether lysine demethylation has gene-specific regulatory functions in lower eukaryotes.

Here we perform a detailed analysis of the demethylation activity of *S. cerevisiae* Kdm5 both in vitro and in vivo. (Note that YJR119c has been termed Jhd2 in recent literature, but we will refer to this protein as Kdm5 in accordance with a new nomenclature system for histone-modifying enzymes [Tony Kouzarides, personal communication]). We investigate whether Kdm5 is involved in regulating H3K4 methylation levels at specific genes. Our results provide evidence for a role of the enzyme in both modulating the level of K4 methylation during the peak of transcription

and removing the modification during attenuation as full gene repression is reestablished.

MATERIALS AND METHODS

Yeast strains and plasmids. Yeast strains used in this study are listed in Table S1 in the supplemental material. Gene deletions were performed as described previously (Longtine, M.S., 1998). Recombinant Kdm5 was created by cloning the *KDM5* open reading frame (ORF) using a TOPO TA cloning kit (Invitrogen). An Expand high-fidelity PCR system (Roche) was used to amplify and subclone the cDNA of *KDM5* into a modified pFAST Bac HTA baculovirus vector containing N-terminal FLAG and six-His tags. The *KDM5* ORF, with a 500-nucleotide upstream sequence and a 250-nucleotide downstream sequence, was similarly cloned using a TOPO TA cloning kit (Invitrogen) and subcloned into pRS314 using an Expand high-fidelity PCR system (Roche). FLAG tagging and amino acid substitutions were made using a QuickChange kit (Stratagene).

Affinity purification of recombinant Kdm5. Flag-His-Kdm5 was expressed in 500 ml of baculovirus-infected Sf21 insect cells and harvested at 72 h (Wistar Institute Protein Expression Core). Cells were lysed with 50 ml insect lysis buffer KCl250 (50 mM HEPES, pH 8.0, 250 mM KCl, 10% glycerol, 0.1% NP-40, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], protease inhibitors), sonicated, and incubated overnight at 4°C with 200 fl mouse anti-Flag (a-Flag) agarose beads (Sigma). Beads were washed five times with 20x volume insect wash buffer KCl500 (50 mM HEPES, pH 8.0, 500 mM KCl, 10% glycerol, 0.1% NP-40, 1 mM EDTA, 0.5 mM PMSF, protease inhibitors) and once with 20x volume demethylation buffer (50 mM HEPES, pH 8.0, 5%

glycerol). The recombinant protein was eluted with 0.5 fg/fl1x Flag peptide in demethylation buffer.

In vitro demethylation reaction and Western analysis. Bulk calf thymus his-tones (4 fg; Sigma) or H3 peptides (100 ng; Upstate) were incubated with the indicated amounts of recombinant proteins in histone demethylase assay buffer [5% glycerol, 50 mM HEPES K, pH 8.0, 2 mM ascorbate, 1 mM a-ketoglutarate, 100 fM Fe(II), 0.2 mM PMSF, protease inhibitors] in a final volume of 11 flat 37°C for 5 h. Incubation products were analyzed by sodium dodecyl sulfatepolyacrylamide gel electrophoresis and Western blotting. Mixtures from the reactions that used histones and peptides as substrates were electrophoresed on 4 to 20% Tris-glycine and 12% NuPAGE gels, respectively (Gallagher, S.R., 2006; Laemmli, U.K., 1970), transferred to PVDF membranes, and probed with methyl-specific antibodies (Gallagher, S., 2004). An anti-H3 antibody served as a loading control. The following antibodies were used for the Western blotting: H3K4me1 (ab8895; Abcam), H3K4me2 (ab7766; Ab-cam), H3K4me3 (07-473; Upstate), H3 (ab1791; Abcam), H3K36me3 (ab9050; Abcam), H3K79me2 (ab3594; Abcam), and FLAG-horseradish peroxidase (A8592; Sigma). For Western analysis on global protein levels, whole-cell extracts were electrophoresed on 4 to 20% Tris-glycine gels, transferred to 0.2-fm nitrocellulose membranes, and probed with the following antibodies: anti-Set1 (from A. Shilatifard, Stowers Institute, MO), anti-FLAG–horseradish peroxidase (M2; Sigma), and anti-H3 (ab1791; Abcam).

ChIP and GAL1 and SUC2 inductions. Chromatin immunoprecipitation (ChIP) experiments were carried out as described before (Burke, D., 2000; Henry, K.W., 2003). For *GAL1* inductions, cells were grown in yeast extract-peptone-dextrose (YPD) overnight, diluted in yeast extract-peptone-raffinose to mid-log phase, and grown for 2 to 3 h. Galactose was added to the media to a final concentration of 2%, and 2 h later glucose was added to a final concentration of 2%. For *SUC2* inductions, cells were grown in YPD overnight, diluted to mid-log phase in the morning, and grown for 2 h. Cells were spun down, washed in yeast extract-peptone plus 0.05% glucose, and resuspended in the low-glucose media. Two hours later, glucose was added to the media for a final concentration of 2%. Cells were cross-linked with 1% formaldehyde for 5 min (histone ChIP) or 20 min (factor ChIP). Protein (1 to 2 mg) was immunoprecipitated as described previously (Henry, K.W., 2003). Antibodies used for ChIP were anti-H3 (ab1791; Abcam), anti-H3 monomethyl K4 (ab8895; Abcam), anti-H3 dimethyl K4 (ab7766; Abcam), anti-H3 trimethyl K4 (ab8580; Abcam), and anti-Set1 (from A. Shilatifard, Stowers Institute, MO). Inputs (1:100 dilutions) and eluates were amplified using an ABI 7900HT fast thermal cycler (Applied Biosystems) and the primer pairs described in Table S2 in the supplemental material. Each PCR consisted of 10 fl SYBR green dye I (Applied Biosystems), 0.1 fl forward primer (10 fM), 0.1 fl reverse primer (10 fM), 4.8 flH2O, and 5 fl sample. The PCRs went through a program consisting of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was run in triplicate, and average values of eluates were normalized to average values of inputs (relative IP) and then further normalized to the relative IP of an untranscribed region of DNA (the IntV region).

RNA analysis. Cells were grown to mid-log phase and 5 to 10 ml of cultures harvested for RNA analysis. Total RNA was prepared by hot acidic phenol extraction as described before (Collart, M.A., 1996). RNA was reverse transcribed with TaqMan reverse transcription reagents (Applied Biosystems) by using random hexamer primers according to the manufacturer's instructions. The resulting cDNA was amplified in real time using an ABI 7900HT fast thermal cycler (Applied Biosystems) with the primer pairs described in Table S2 in the supplemental material. Each PCR consisted of 10 fl SYBR green dye I (Applied Biosystems), 0.1 fl forward primer (10 fM), 0.1 fl reverse primer (10 fM), 4.8 flH2O, and 5 fl sample. The PCRs went through a program consisting of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The data were normalized to amplification of 18S ribosomal cDNA.

Antibody production. The recombinant N-terminally truncated Set1 protein (rSet1) was expressed in *Escherichia coli*. The pMCSG7 plasmid vector was used for protein expression (Stols, L., 2002) to produce a fusion protein with polyhistidine tags along with the Set1 protein coding sequence. The construction of the expression vector was carried out by PCR using primers 5' TACTTCCAATTCAATGCTa tgAGCACATATACTCCTACCGTCA 3' and 5' TTATCCACTTCCAATGtca GTTCAAGAAACCTTTACAATTAGGTG 3' (lowercase letters represent the positions of the start and stop codons). One milligram of purified rSet1 protein was injected into rabbits and guinea pigs (Pocono Rabbit Farm & Laboratory) (protocol for antibody production at http://www.prfal.com/protocol.php).

RESULTS

Kdm5 has specific demethylase activity towards all methylated states of H3K4.

The five *S. cerevisiae* predicted ORFs bearing homology to JmjC catalytic domains were compared to Jarid1d, a histone demethylase in mammals that targets H3K4me3 and H3K4me2 (Lee, M.G., 2007). Within the JmjC domain and over the entire protein, Kdm5 (YJR119c) has the highest identity to Jarid1d (Fig. 1A; see also Fig. S1 in the supplemental material). Further, Kdm5 (like Jarid1d) has JmjN, BRIGHT, and PHD domains in colinear arrangement with Jarid1d, which is not the case for the other yeast jumonji proteins (Fig. 1A; see also Fig. S1 in the supplemental material). These similarities prompted us to test whether Kdm5, like Jarid1d, is a histone H3K4 demethylase.

FLAG-tagged Kdm5 was expressed via baculovirus infection of Sf21 insect cells. After 72 h of infection, cells were lysed and recombinant protein purified on FLAG antibody beads, followed by FLAG peptide elution, gel electrophoresis, and Colloidal Blue staining. The results showed one band of expected size for Kdm5 (Fig. 1B), which was confirmed by Western blotting with FLAG antibody (data not shown). The protein was tested in a demethylase assay using calf thymus histones as substrates, under conditions that were used previously to test Jarid1d (Lee, M.G., 2007). The assay with the yeast protein resulted in a substantial reduction in H3K4me3 and H3K4me2 levels in a dose-dependent manner and a slight reduction in H3K4me1 levels (Fig. 1C). However, no changes in K36me3 and K79me2 levels were observed (Fig. 1C), which is in accordance with other reports showing no activity of this enzyme towards H3 methylated on K36 and K79 (Liang, G., 2007). This profile of activity was similar to that of Jarid1d, used as a control for these reactions (Fig. 1C).

In order to investigate the importance of the different domains of Kdm5, we created point mutations at conserved residues in either the PHD domain (D254A) or the JmjC domain (H427A). Recombinant Kdm5 carrying either of these mutations was expressed in insect cells and yielded amounts of protein equal to that of the wild-type protein (Fig. 1B). When tested in the demethylase assay, the recombinant protein carrying a mutation in the PHD domain (D254A) yielded results similar to those for the wild-type protein (Fig. 1D). However, the JmjC domain mutant (the H427A mutant) showed no activity towards the histone substrates methylated on H3K4 (Fig. 1D), suggesting that the enzymatic activity of Kdm5 is indeed dependent on the conserved sequence of the JmjC domain.

Although these assays showed only a minor reduction in H3K4me1 levels, the full activity of the enzyme towards this substrate could be masked by the conversion of H3K4me3 and H3K4me2 into the H3K4me1 form. Therefore, we performed the demethylase assay with histone H3 peptides containing the three forms of methylated K4. The reactions where the substrate was either H3K4me3 or H3K4me2 resulted in an accumulation of H3K4me1, but the reaction with H3K4me1 as a substrate resulted in an almost complete reduction in me1 signal (Fig. 1E). Again, the recombinant protein with the H427A mutation did not show any activity towards the methylated peptides (Fig. 1E).

Kdm5 affects H3K4 methylation levels during active transcription of the GAL1 gene and has modest effects on GAL1 expression.

We analyzed the function of Kdm5 in vivo. A strain bearing a deletion of the gene was generated and tested for effects on H3K4 methylation. Histones were prepared by acid

extraction from the parental and deletion strains and analyzed by quantitative Western blotting using K4 methylation-specific antibodies. No clear differences in the levels of mono-, di-, or trimethylation were detected, and an antibody for unmodified histone was used as a comparison (data not shown).

Although global methylation levels are not altered substantially by deletion of *KDM5*, methylation may be affected on specific genes. We examined the *GAL1* gene under conditions where RNA is not transcribed (raffinose) or transcribed at high levels (galactose) (Fig. 2A). ChIP analysis using highly specific antibodies (see Fig. S2 in the supplemental material) was carried out for H3K4 methylation at the *GAL1* gene under these conditions. We examined mono-, di-, and trimethylation levels relative to the IntV region and normalized to unmodified H3 levels. In the wild-type strain, K4 monomethylation increases twofold at the 5' end of the ORF in galactose, whereas, as expected (Ng, H.H., 2003), both di- and trimethylation strongly increase in galactose (approximately 10-fold) (Fig. 2B). We examined K4 methylation levels in the *KDM5* deletion relative to the wild type in galactose and found substantial increases for mono and dimethylation (more than fourfold) and lower increases for trimethylation (less than twofold) (Fig. 2B). We analyzed the 3' end of the ORF and observed no increases for mono, di-, or trimethylation in the wild-type strain in galactose but increases in all three states in the *KDM5* deletion strain relative to the wild type (see Fig. 5A).

We examined *GAL1* RNA levels in the *KDM5* deletion strain in galactose to determine whether the increase in methylation is reflected in higher transcription. RNA levels are slightly higher in the absence of Kdm5 (Fig. 2C). We speculated that there might be an additive effect on RNA levels among the *S. cerevisiae* jumonji domain proteins. To test this, we prepared a double disruption of *KDM2* (*JHD1*) and *KDM5*. We

chose *KDM2* because it was shown previously to have demethylase activity towards H3 methylated on K36 (Fang, J., 2007; Tsukada, Y., 2006), a distinct activation-linked H3 methylation (Henry, K.W., 2003). We found that the double disruption showed a greater increase in RNA levels than either single disruption (Fig. 2C), an effect that was observed at various times of galactose induction (data not shown).

Kdm5 affects Set1 recruitment during active transcription of the GAL1 gene.

The Set1 enzyme carries out all H3K4 methylation in *S. cerevisiae*, including mono-, di-, and trimethylation (Briggs, S.D., 2001; Krogan, N.J., 2002; Nagy, P.L., 2002; Roguev, A., 2001; Santos-Rosa, H., 2002). Set1 is a component of the protein complex COMPASS, composed of intrinsic components that regulate its relative abilities to mono-, di-, and trimethylate K4 (Morillon, A., 2005; Nagy, P.L., 2002; Roguev, A., 2001; Schneider, J., 2005). Higher levels of methylation at *GAL1* in the *kdm5* mutant led us to examine the level of the Set1 enzyme at the gene. We used an antibody highly specific for Set1 as shown by Western analysis of whole-cell yeast extracts and ChIP assay (Fig. 3A). ChIP assays showed that Set1 levels increased more than twofold in the *KDM5* deletion strain compared to the wild type in galactose, both at the 5' ORF (Fig. 3B) and the 3' ORF (see Fig. 5C, left) of *GAL1*. To test whether the lower Set1 recruitment in the wild-type strain was dependent on the demethylase activity of Kdm5, we performed the same ChIP experiments with two additional strains consisting of the *KDM5* deletion strain reconstituted with either a wild-type copy of *KDM5* or a copy of *KDM5* carrying a mutant JmjC domain. Both the wild-type and the mutant copy were FLAG tagged and were found to express at equal levels judging by FLAG Western blot analysis of whole-cell extracts (Fig. 3C). The FLAG-tagged wild-type copy complemented the deletion

strain, as it resulted in the same low levels of Set1 recruitment during galactose induction as the wild-type parental strain (Fig. 3D). However, the deletion strain containing the mutant JmjC domain protein showed as high levels of Set1 as the deletion strain alone (Fig. 3D), suggesting that the demethylase activity of Kdm5 is involved in the regulation of Set1 recruitment during *GAL1* activation. This effect is not due to global changes in Set1 protein levels as they were determined to be equal in all strains used for these ChIP experiments (Fig. 3E).

Kdm5 affects H3K4 methylation levels during shutdown of GAL1 transcription.

Transcription of *GAL1* shuts down as the cells are shifted from galactose media into repressive glucose (Fig. 2A). We tested whether demethylation of H3K4 correlates with this attenuation phase of gene expression. When the wild-type strain is switched from galactose growth to glucose, trimethylation is reduced, dimethylation remains constant, and monomethylation increases slightly at the 5' end of the gene (Fig. 4A). Interestingly, the reduction in dimethylation and especially in trimethylation is significantly delayed in the absence of Kdm5 (Fig. 4A). We found that the wild-type and *KDM5* deletion strains grew at the same rates (data not shown); hence, the methylation difference detected in the two strains during return to the repressive state is not the result of altered replication. We examined Set1 levels in glucose conditions and found that the levels decreased in the absence of Kdm5, just as in the wild-type strain (Fig. 4B). The 3' end of the gene also showed an increase in methylation but no increase in Set1 levels (Fig. 5B and C, right). Thus, persistence of high methylation without Kdm5 is not due to Set1 remaining bound to the *GAL1* gene. To determine whether the effects on methylation levels were specifically due to Kdm5 enzymatic activity, we performed these ChIP experiments with

the two additional strains used previously for the Set1 ChIP, containing either a wild-type copy of *KDM5* or a copy of *KDM5* carrying a mutant JmjC domain. All four strains were grown during galactose-inducing conditions followed by glucose-repressive conditions, and ChIP analysis was carried out as described above. When focused on repressive conditions for *GAL1*, where trimethylation of H3K4 is reduced in a wild-type strain, we saw similar trimethylation levels in the *KDM5* deletion strain carrying a FLAG-tagged wild-type copy of *KDM5* (Fig. 4C). However, the mutant copy of *KDM5* resulted in higher trimethylation levels, comparable to the levels seen in the deletion strain (Fig. 4C). These results add further support to our hypothesis that Kdm5 has demethylase activity in vivo and that this activity is involved directly in gene regulation.

Kdm5 lowers H3K4 trimethylation levels during shutdown of SUC2 transcription.

We examined H3K4 methylation levels at other genes in the *KDM5* deletion strain. First, we tested constitutive genes, such as *PMA1* and *ADH1*, but could not detect any differences in methylation levels between the wild-type and the deletion strains (data not shown). Next we examined another inducible gene, *SUC2*. *SUC2* RNA levels increased during the induction, but there was no significant difference in transcription between the wild-type and the deletion strains (data not shown). ChIP analysis of H3K4 trimethylation levels at the 5' end of the gene showed a substantial decrease in the wild-type strain from the induced state back to the repressed state of *SUC2* transcription, whereas the *KDM5* deletion strain showed only a very slight decrease (Fig. 6). These results show that Kdm5 effects on K4 methylation levels are not restricted to the *GAL1* gene.

DISCUSSION

Here we report that the protein encoded by *KDM5* is a histone demethylase and shows substrate specificity for histone H3K4 in vitro. Kdm5 specifically reverses all methylated states of K4 but not other H3 methylation states (K36me3 and K79me2) (Fig. 1C and D). A mutation in the JmjC domain of Kdm5 abrogates this demethylase activity towards methylated H3K4, whereas a mutation in the PHD domain does not (Fig. 1D).

We have observed Kdm5-dependent H3K4 demethylation in vivo within the *GAL1* and *SUC2* ORFs. K4 is methylated by the Set1 component of the COMPASS complex, and the relative levels of mono-, di-, and trimethylation are established by regulation of COMPASS (Schneider, J., 2005). While monomethylation does not increase during gene activation, both di-and trimethylation increase when *GAL1* is induced by growth in galactose media. The trimethylated form of H3K4 is known to be focused specifically at the 5' end of genes (Santos-Rosa, H., 2002), and, via PHD domain interactions, it may help to recruit complexes involved in gene activation (Taverna, S.D., 2006).

We observed three consequences of deleting Kdm5, manifesting in altered amounts and locations of K4 methylation within the *GAL1* ORF. One normal function of Kdm5 is to prevent high levels of Set1 recruitment during maximally active gene transcription (Fig. 3B). The elevated levels of Set1 recruitment in the *KDM5* deletion strain result in abnormally high levels of mono-and dimethylated H3K4 (Fig. 2B). The result is an altered balance of the methylation states, where the relative increase in trimethylation is reduced compared to the increases in mono-and dimethylation. This function of Kdm5 is dependent upon its enzymatic activity, as the catalytic mutant version of Kdm5 shows the same high levels of Set1 recruitment at the *GAL1* 5' ORF

region as the *KDM5* deletion strain (Fig. 3D). One possible explanation for this effect might be that the loss of Kdm5 activity results in the recruitment of a specific form of COMPASS, one that produces primarily di-and monomethylated H3K4. For instance, it has been shown that a strain lacking the COMPASS subunit Cps60 or Cps40 results in normal global levels of H3K4 di-and monomethylation but almost nonexistent levels of H3K4 trimethylation (Schneider, J., 2005).

Second, during the attenuation phase following induced transcription, the level of trimethylated K4 is reduced, finally falling to levels characteristic of the fully repressed gene. It appears that the loss of methylation is an active process mediated by Kdm5, because deletion of the demethylase results in delayed reduction of the methylation (Fig. 4A). This is further supported by analysis of a strain where Kdm5 bears a mutant JmjC domain, which shows methylation levels comparable to those for the *KDM5* deletion strain (Fig. 4C). In contrast to the events that occur during *GAL1* induction, during repression of this gene, the Set1 recruitment in the *KDM5* deletion strain is not higher than the recruitment levels in the wild-type strain. This suggests that Kdm5 is actively demethylating H3K4 at the *GAL1* 5' ORF during the transcriptional repression phase. Another inducible gene, *SUC2*, also shows a delayed reduction in trimethylation levels in the deletion strain during attenuation. It may be that Kdm5 functions primarily at inducible genes, since none of the constitutively active genes tested showed differences in methylation levels between the *KDM5* deletion and wild-type strains. In mammalian cells, low methylation levels in repressed genes have been shown to be maintained by JARID demethylases (Christensen, J., 2007; Klose, R.J., 2007; Lee, M.G., 2007), but it was not known previously that there is active demethylase-dependent reduction of H3K4 trimethylation levels during shutdown of transcription. However, we cannot conclude that

Kdm5 is directly responsible for this demethylation event since we were not able to detect Kdm5 protein at the *GAL1* ORF by ChIP. The *S. cerevisiae* and the *Schizosaccharomyces pombe* JmjC proteins appear to be refractory to ChIP, as other groups report similar difficulty (Huarte, M., 2007; Kim, T., 2007). This may be due to transient association of the enzymes with chromatin or to the enzymes functioning on histones as octamers while not tightly associated with DNA.

A third function of Kdm5 is observed at the 3' end of the gene. Normally, there is no apparent increase in any methylation state or Set1 levels during gene activation, whereas in the absence of Kdm5 there is an increase in Set1 and in all methylation states relative to those for the wild type (Fig. 5).

Our analysis thus reveals that Kdm5 has multiple roles in regulating K4 methylation. At the 5' end of the *GAL1* ORF, Kdm5 establishes relative methylation levels, limits methylation levels during activation, and functions to reduce methylation levels during attenuation of transcription. At the 3' end of the gene, Kdm5 prevents methylation. It thus appears that reversible lysine methylation of histones is conserved through evolution, and this investigation of *S. cerevisiae* reveals novel gene-specific regulatory functions of demethylation.

ACKNOWLEDGEMENTS

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Figure 1. Kdm5 demethylates H3K4 mono-, di-, and trimethyl. (A) Schematic representation of JARID1d and the five closest *S. cerevisiae* homologues, including Kdm5. Sequence identities of the JmjC domains of the five proteins to the JARID1d JmjC domain are indicated. aa, amino acids. (B) Colloidal Blue staining of recombinant Kdm5. Flag-His-Kdm5 (wild type, H427A mutant [JmjC domain], and D254A mutant [PHD domain]) was purified from Sf21 cells using an α-Flag column and eluted in demethylation buffer using Flag peptides. The purity of the enzymes was determined by electrophoresis of 4µL of the protein on a 4 to 20%Tris-glycine gel, followed by staining with Colloidal Blue. (C) Demethylation reactions were performed with 4µg of bulk calf thymus histones and no enzyme, recombinant JARID1d (a known demethylase), or increasing amounts of recombinant Kdm5. Reaction mixtures were electrophoresed on a 4 to 20% Tris-glycine gel, transferred to PVDF membranes, and probed with methyl-specific antibodies. An anti-H3 antibody served as a loading control. Amounts of recombinant protein added are indicated above the lanes. Antibodies used are indicated to the left of each blot. (D) Demethylation assay comparing recombinant wild-type Kdm5 (WT) and the two mutant Kdm5 enzymes (H427A and D254A). The reaction was carried out and analyzed as described for panel C. Approximately 3µg of recombinant protein was used for each reaction. (E) Demethylation reactions were performed with 100ng of histone H3 peptides containing mono-, di-, or trimethylated lysine 4 and no enzyme, 3µg of recombinant wild-type enzyme (WT), or 3µg of recombinant mutant enzyme (H427A). Reaction mixtures were run on 12% NuPAGE gels, transferred to PVDF membranes, and probed with methyl-specific antibodies. Enzymes and peptides used are indicated above the lanes. An anti-FLAG antibody indicated the relative amounts of recombinant enzymes. Antibodies used are indicated to the left of the blots.

Figure 2. RNA and histone H3 K4 methylation levels during activation of *GAL1*. (A) *GAL1* RNA levels in wild-type strain during galactose induction and return to growth in glucose. RNA was extracted from cell pellets collected after 2 h of growth in raffinose (RAFF)-containing media, 2 h of growth in galactose (GAL), and 15 min of growth in glucose (GLU). Quantitative reverse transcription-PCR experiments were performed with total RNA to assess the transcript levels of *GAL1*. *GAL1* expression levels after normalizing each signal to those obtained from the 18S rRNA locus are shown and presented as levels of induction, with *GAL1* levels in raffinose set to 1. Error bars show standard deviations (SD). (B) Histone H3 K4 methyl ChIP in wild-type and *KDM5* deletion strains, followed by quantitative PCR analysis at the *GAL1* 5' ORF. Samples were taken after 2 h of growth in raffinose and 2 h of growth in galactose. Each graph is a representative of several ChIP experiments. Each data point represents an average of two to three quantitative PCR analyses. Error bars show SD of quantitative PCR analysis. Specificity of the antibodies used for ChIP is shown in Fig. S2 in the supplemental material. Data are shown as percentages of input (relative IP) after being normalized to relative IP of the IntV region and again normalized to total histone H3 ChIP signals. The normalized signal for the wild-type (WT) strain in raffinose media was set to 1. (C) *GAL1* RNA levels analyzed in samples taken after 30 min of growth in galactose media for the indicated strains. RNA was analyzed by reverse transcription followed by real-time amplification. *GAL1* RNA levels were normalized to 18S RNA levels and wild-type *GAL1* levels set to 1. Error bars show SD of quantitative PCR analysis.

Figure 3. Set1 levels during activation of *GAL1*. (A) Specificity of α-Set1 antibody. (Top) Western blot analysis of increasing amounts of whole-cell extracts from either a wild-type (WT) strain or a *SET1* deletion strain. (Bottom) Set1 ChIP with two negative controls, a *SET1* deletion strain and rabbit immunoglobulin G (NoAb). (B) Set1 ChIP in a wild-type strain and a *KDM5* deletion strain, followed by quantitative PCR analysis at the *GAL1* 5' ORF. Samples were taken after 2 h of growth in raffinose (RAFF) and 2 h of growth in galactose (GAL) and analyzed as described in the legend for Fig. 2B. Error bars show standard deviations of quantitative PCR analysis. Data are shown as percentages of input (relative IP) after being normalized to relative IP of the IntV region. The normalized signal for the wild-type strain in raffinose media was set to 1. (C) Expression levels of FLAG-tagged wild-type and H427A E429A mutant KDM5. Western blot analysis of whole-cell extracts from the indicated strains. The upper panel shows the FLAG Western blot, and the lower panel shows an H3 Western blot as a loading control. (D) Set1 ChIP in the indicated strains, followed by quantitative PCR analysis at the *GAL1* 5' ORF. Samples were taken after 2 h of growth in galactose and analyzed as described for panel B. The normalized signal for the wild-type strain was set to 1. *kdm5*Δ+KDM5, *KDM5* deletion strain with a FLAG-tagged wild-type copy of *KDM5*; *kdm5*Δ+kdm5 H427AE429A, *KDM5* deletion strain with a FLAG-tagged H427A E429A mutant copy of *KDM5*. (E) Expression levels of Set1. Western blot analysis of whole-cell extracts from the indicated strains. The upper panel shows the Set1 Western blot, and the lower panel shows an H3 Western blot as a loading control.

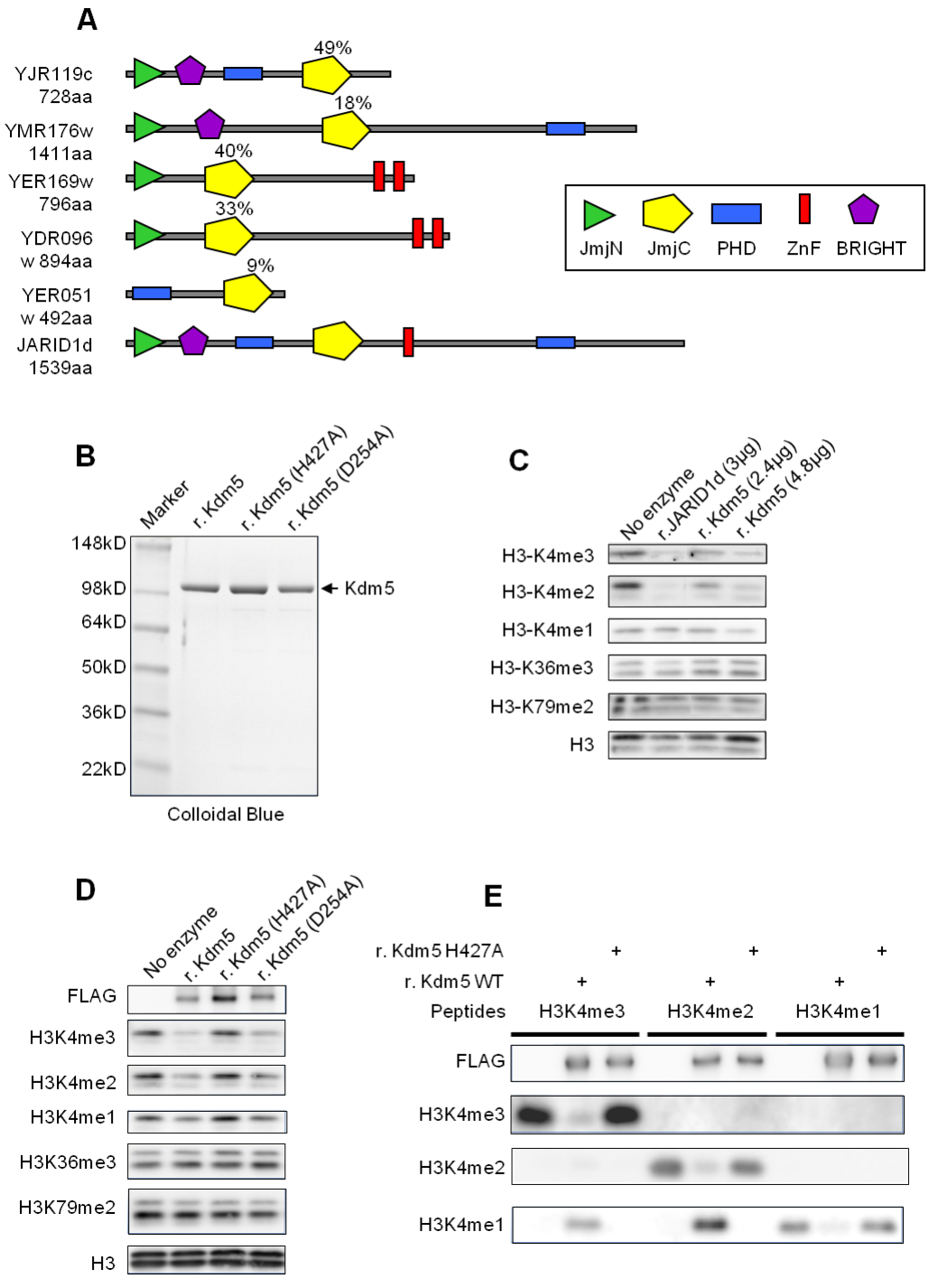
Figure 4. Histone H3 K4 methylation and Set1 levels during repression of *GAL1*. (A) Histone H3 K4 methyl ChIP in wild-type (WT) and *KDM5* deletion strains, followed by quantitative PCR analysis at the *GAL1* 5' ORF. Samples were taken after 75 min and 280 min of growth in glucose (GLU) and analyzed as described in the legend for Fig. 2B. (B) Set1 ChIP in the wild-type strain and the *KDM5* deletion strain, followed by quantitative PCR analysis at the *GAL1* 5' ORF. Samples were taken after 75 min and 280 min of growth in glucose and analyzed as described in the legend for Fig. 3B. (C) Histone H3 K4 trimethyl ChIP in the indicated strains, followed by quantitative PCR analysis at the *GAL1* 5' ORF. Samples were taken after 280 min of growth in glucose and analyzed as described in the legend for Fig. 2B. *kdm5*∆+KDM5, *KDM5* deletion strain with a FLAG-tagged wild-type copy of *KDM5*; *kdm5* ∆+kdm5 H427AE429A, *KDM5* deletion strain with a FLAG-tagged H427A E429A mutant copy of *KDM5*.

Figure 5. Histone H3 K4 methylation and Set1 levels at *GAL1* 3' ORF. (A) Histone H3 K4 methyl ChIP in wild-type and *KDM5* deletion strains during activation of *GAL1*. Samples were taken after 2 h of growth in raffinose (RAFF) and 2 h of growth in galactose (GAL). Each graph is a representative of several ChIP experiments. Each data point represents an average of two to three quantitative PCR analyses. Error bars show standard deviations of quantitative PCR analysis. Data are shown as percentages of input (relative IP) after being normalized to relative IP of the IntV region and again normalized to total histone H3 ChIP signals. The signal for the wild-type (WT) strain in raffinose media was set to 1. (B) Histone H3 K4 methyl ChIP in wild-type and *KDM5* deletion strains during repression of *GAL1*. Samples were taken after 75 min and 280

min of growth in glucose (GLU) and analyzed as described above. (C) Set1 levels during activation and repression of *GAL1*. (Left) Samples were taken after 2 h of growth in raffinose and 2 h of growth in galactose and analyzed as described in the legend for Fig. 3B. (Right) Samples were taken after 75 min and 280 min of growth in glucose and analyzed as described in the legend for Fig. 3B.

Figure 6. Histone H3 K4 methylation levels during activation and repression of *SUC2*. Histone H3 K4 trimethyl ChIP in wild-type (WT) and *KDM5* deletion strains, followed by quantitative PCR analysis at the *SUC2* 5_ ORF. Samples were taken after 2 h of growth in low glucose (GLU) (0.05%) and both 15 and 30 min of growth in YPD. Each data point represents an average of two to three quantitative PCR analyses. Error bars show standard deviations of quantitative PCR analysis. Data are shown as percentages of input (relative IP) after being normalized to relative IP of the IntV region and again normalized to total histone H3 ChIP signals. The normalized signal for the wild-type strain in low-glucose media was set to 1.

Figure 1.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18%
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w 492aa
9%
JARID1d
1539aa
JmjN
JmjC
PHD
ZnF
BRIGHT
B
Marker
r. Kdm5
r. Kdm5 (H427A)
r. Kdm5 (D254A)
148kD
98kD
64kD
50kD
36kD
22kD
Kdm5
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C
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r. Kdm5 (2.4µg)
r. Kdm5 (4.8µg)
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H3-K4me2
H3-K4me1
H3-K36me3
H3-K79me2
H3
D
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r. Kdm5 (H427A)
r. Kdm5 (D254A)
FLAG
H3K4me3
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r. Kdm5 WT
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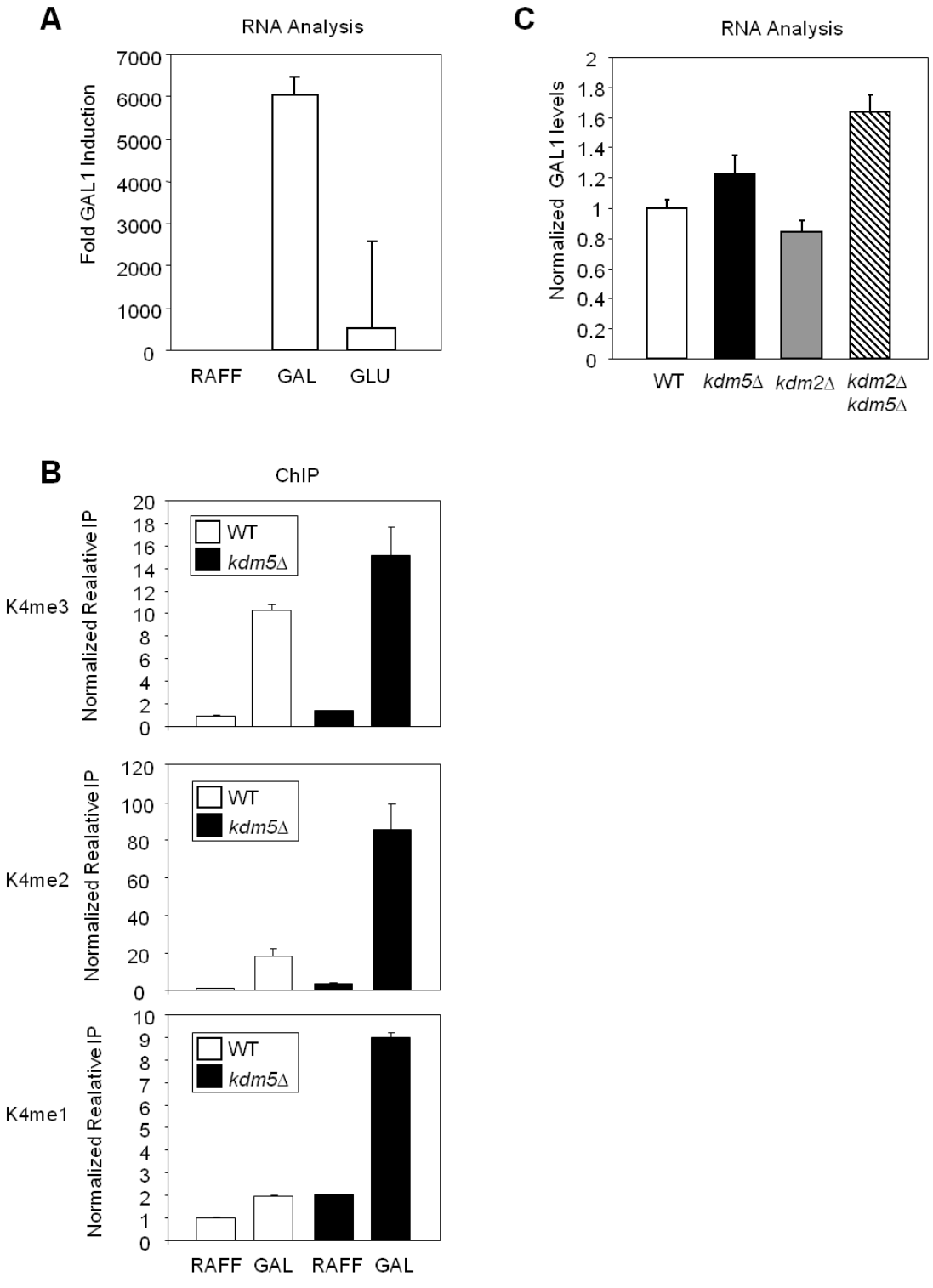
H3K4me1

Figure 2.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5000
4000
3000
2000
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GAL
GLU
C
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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
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kdm5Δ
kdm2Δ
kdm2Δ kdm5Δ
B
ChIP
K4me3
Normalized Realative IP
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18
16
14
12
10
8
6
4
2
0
WT
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K4me2
Normalized Realative IP
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80
60
40
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K4me1
Normalized Realative IP
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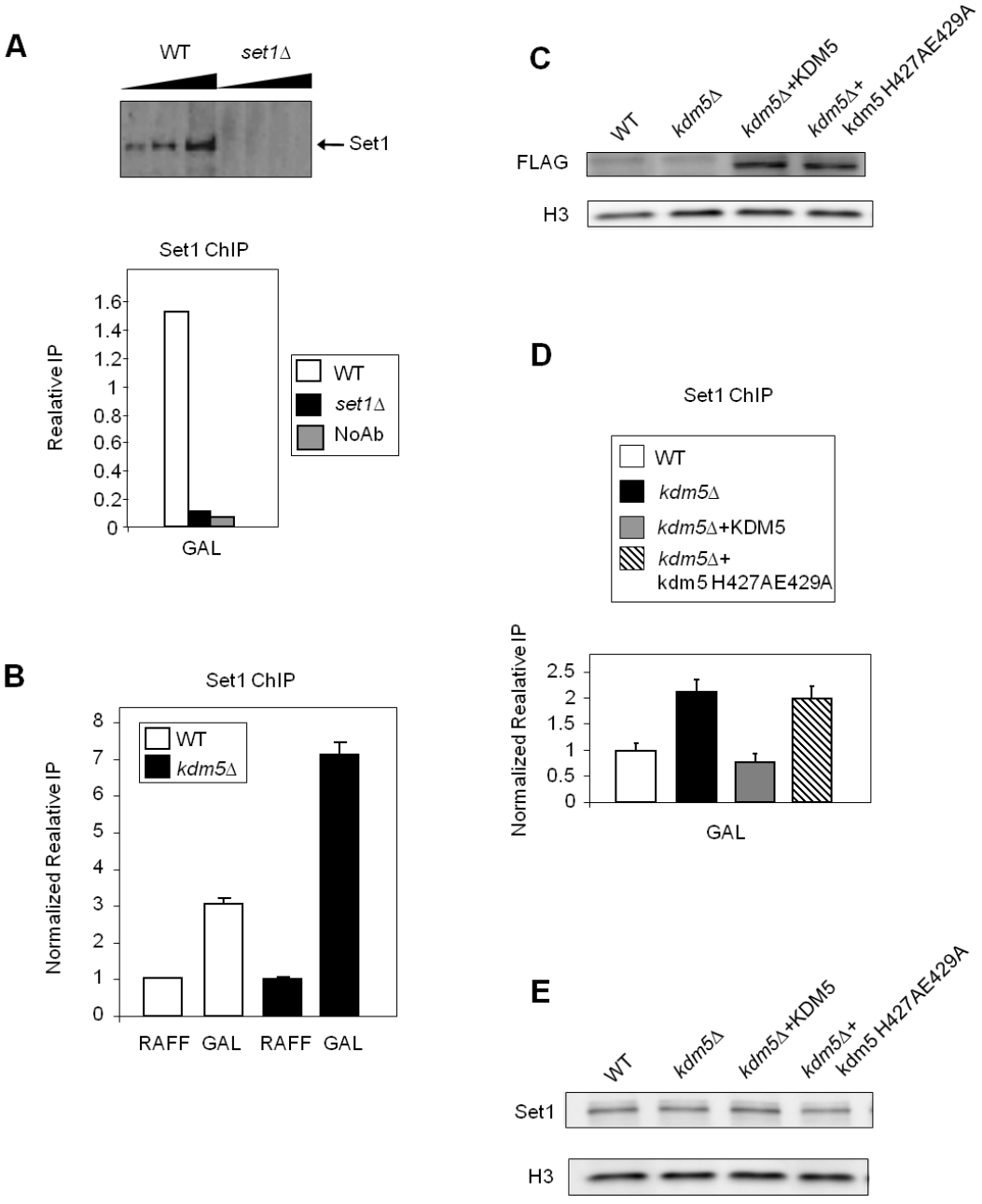
RAFF GAL RAFF GAL

Figure 3.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WT
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Set1
Set1 ChIP
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0.2
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GAL
WT
set1Δ
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B
Set1 ChIP
Normalized Realative IP
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7
6
5
4
3
2
1
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C
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kdm5Δ+ kdm5 H427AE429A
FLAG
H3
D
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kdm5Δ+KDM5
kdm5Δ+ kdm5 H427AE429A
Normalized Realative IP
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2
1.5
1
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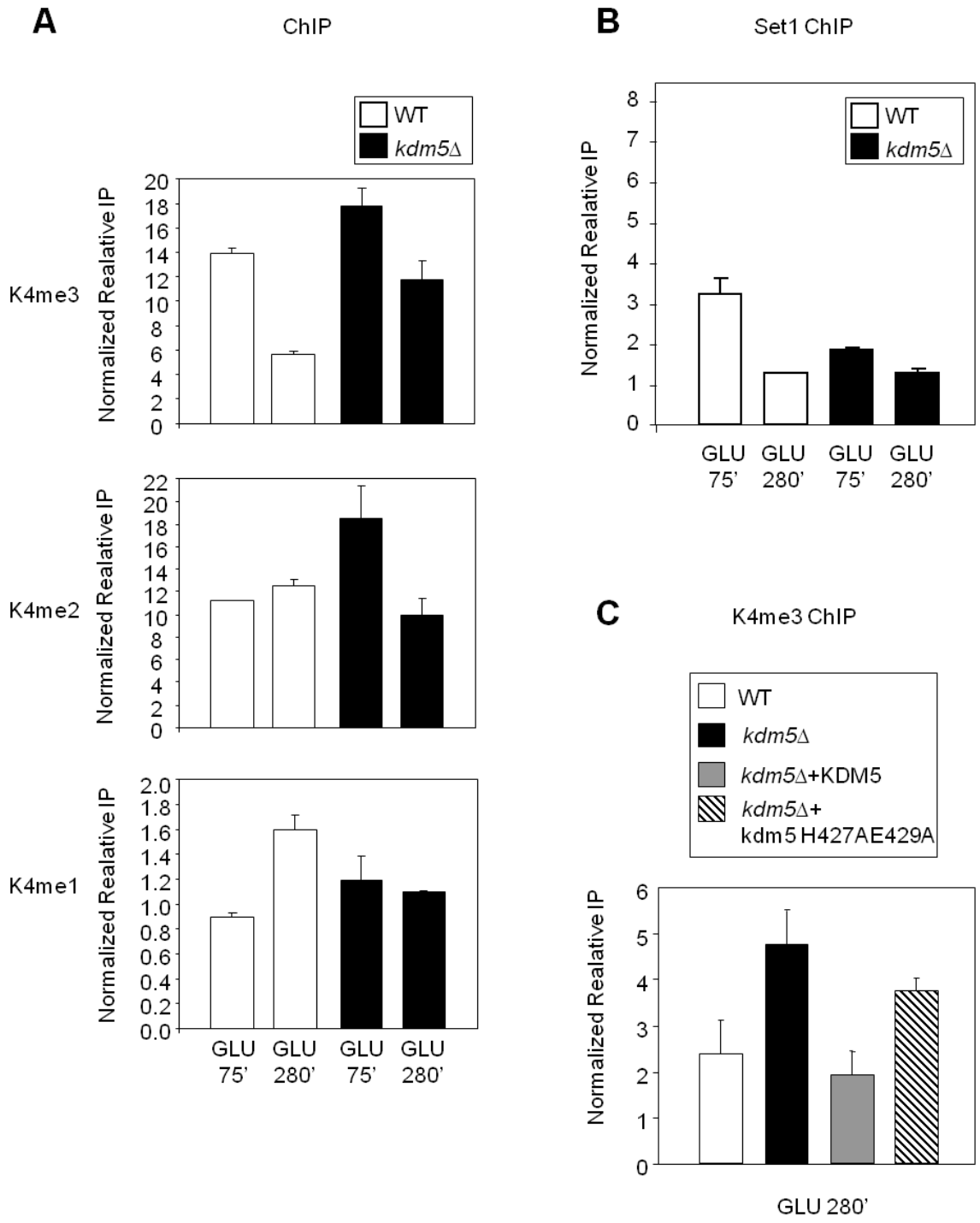
Set1
H3

Figure 4.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K4me3
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K4me1
Normalized Realative IP
GLU 75'
GLU 280'
GLU 75'
GLU 280'
B
Set1 ChIP
WT
kdm5Δ
Normalized Realative IP
GLU 75'
GLU 280'
GLU 75'
GLU 280'
C
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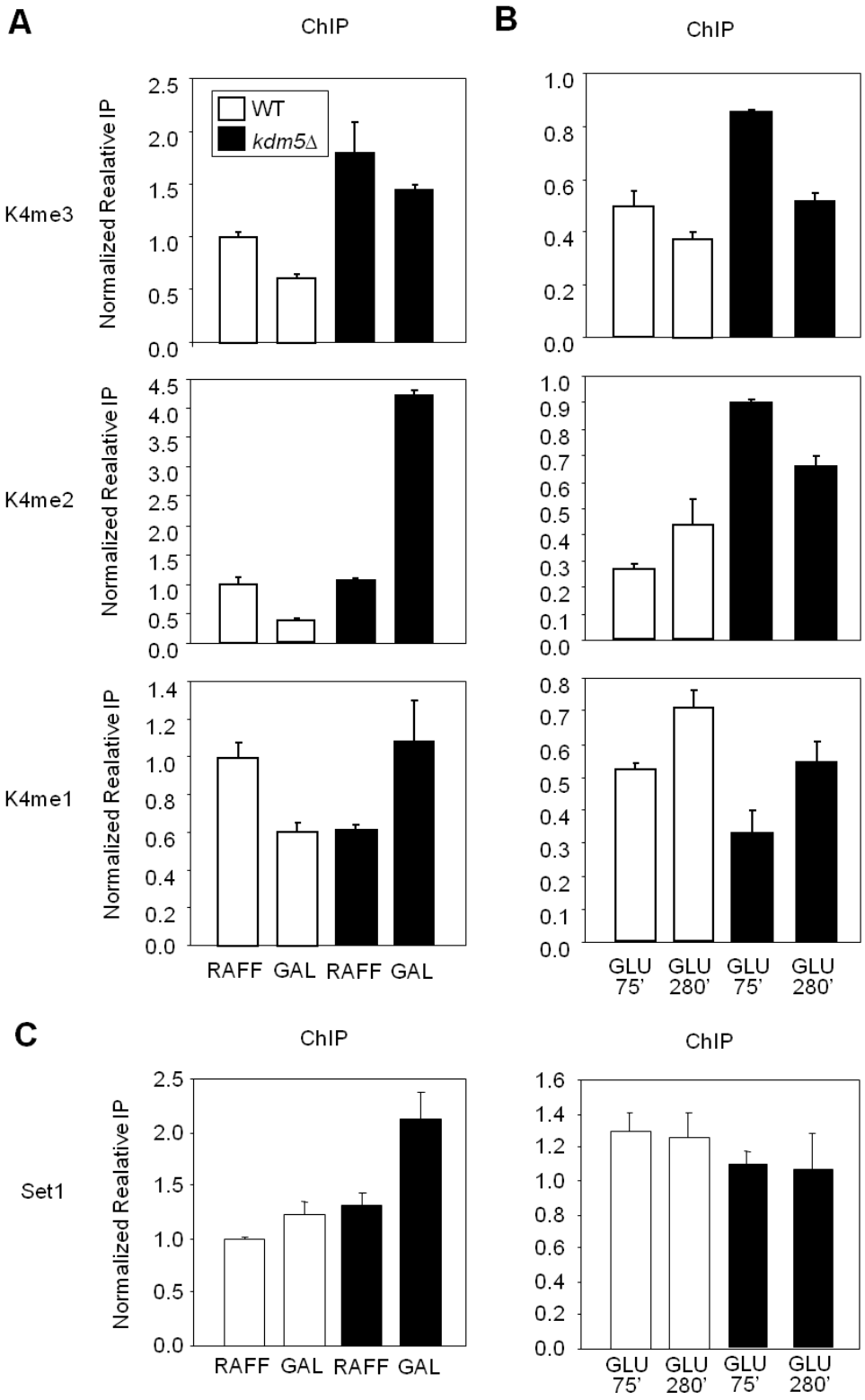
kdm5Δ+ kdm5 H427AE429A
Normalized Realative IP
GLU 280'

Figure 5.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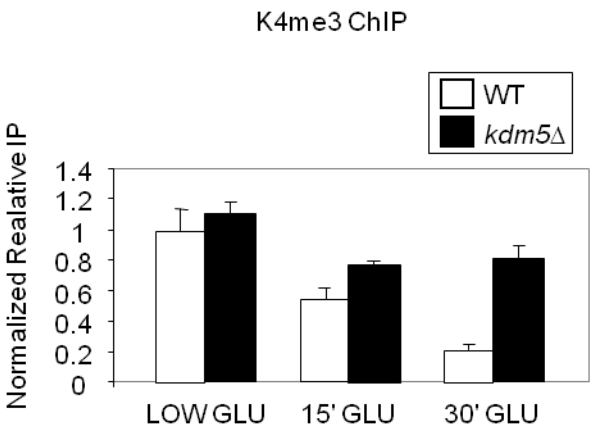
B
ChIP
WT
kdm5Δ
K4me3
K4me2
K4me1
Normalized Realative IP
RAFF GAL RAFF GAL
GLU 75' GLU 280' GLU 75' GLU 280'
C
ChIP
ChIP
Set1
Normalized Realative IP
RAFF GAL RAFF GAL
GLU 75' GLU 280' GLU 75' GLU 280'

Figure 6.


K4me3 ChIP
WT
kdm5Δ
Normalized Realative IP
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1.2
1
0.8
0.6
0.4
0.2
0
LOW GLU
15' GLU
30' GLU

Section 3 Supplementary Materials for Section Two

Supplementary Table 1. Yeast strains used in this study.

Name	Genotype
SB301	Mata his3Δ200 leu2Δ1 ura3-52trp1ΔhisG
YKI127	Mata his3Δ200 leu2Δ1 ura3-52trp1ΔhisG yjr119c::HIS3
YKI113	Mata his3Δ200 leu2Δ1 ura3-52trp1ΔhisG yer051w::KanMX
YKI183	Mata his3Δ200 leu2Δ1 ura3-52trp1ΔhisG yer051w::KanMX yjr119c::HIS3
YKI200	Mata his3Δ200 leu2Δ1 ura3-52trp1ΔhisG set1::HIS3
YKI204	Mata his3Δ200 leu2Δ1 ura3-52trp1ΔhisG pRS314
YKI205	Mata his3Δ200 leu2Δ1 ura3-52trp1ΔhisG yjr119c::HIS3 pRS314
YKI206	Mata his3Δ200 leu2Δ1 ura3-52trp1ΔhisG yjr119c::HIS3 pRS314 FLAG-YJR119C
YKI207	Mata his3D200 leu2D1 ura3-52 trp1DhisG yjr119c::HIS3 pRS314 FLAG-yjr119c h427a,e429a
FY1716	MATa *his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128δ(hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pDM1* [*HHT2-HHF2 CEN-URA3*]
YKI220	MATa *his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128δ(hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 [hht2-k4a-HHF2-CEN-TRP1]*

Supplementary Table 2. Oligonucleotides used in this study.

Name	Sequence (5'-3')	Location
GAL1_5'ORF-F	ACC GGA TTT TGT TGC TAG A	131nt from GAL1 start
GAL1_5'ORF-R	TGT TCA CCA ATT AGA TTG ACT CTA CCA G	178nt from GAL1 start
GAL1_3'ORF-F	GGG TGG TTG TAC TGT TCA CTT GG	177nt from GAL1 stop
GAL1_3'ORF-R	TCT ATG TTG CCA TTT GGG CC	107nt from GAL1 stop
IntV-F	TAA GAG GTG ATG GTG ATA GGC GT	Chr V 9762-9784
IntV-R	CCC TCG GGT CAA ACA CTA CAC	Chr V 9809-9830
18S-F	AAT AAG GGT TCG ATT CCG GAG	RDN18-1 ORF
18S-R	TGG ATG TGG TAG CCG TTT CTC	RDN18-1 ORF
SUC2-5'ORF-F	CATCA ATGACAAACG AAACTAGCG	56nt from SUC2 start
SUC2-5'ORF-R	TGGGTGTGAAGTGGACCAAA	106nt from SUC2 start

Supplementary Figure 1. An alignment of the JmjN, PHD, and JmjC domains of KDM5 and JARID1d. Identical residues highlighted in red. Residues mutated in the JmjC and the PHD domain are in blue and underlined.

Supplementary Figure 2. Specificity of histone antibodies. ChIP of wild-type strain and a strain carrying a K4A mutation in histone H3 grown in raffinose using either the indicated histone specific antibodies (wild-type signal set to 1) or rabbit IgG (No Ab), followed by quantitative PCR analysis. Error bars show s.d. White bar: Wild-type strain. Grey bar: H3K4A strain. Striped bar: ChIP samples of wild-type strain with rabbit IgG (No Ab).

Supplementary Figure 1. An alignment of the JmjN, PHD, and JmjC domains of KDM5 and JARID1d.

```
JmjC Domains
   Jarid1d   VGMVFSAFCWHIEDHWSYSINYLHWGEPKTWYGVPSLAAEHLEEVMKMLT
      Kdm5   IGSLFSTFCWHMEDQYTLSANYQHEGDPKVWYSIPESGCTKFNDLLNDMS

   Jarid1d   PELFDSQPDLLHQLVTLMNPN--TLMSHGVPVVRTNQCAGEFVITFPRAY
      Kdm5   PDLFIKQPDLLHQLVTLISPYDPNFKKSGIPVYKAVQKPNEYIITFPKCY

   Jarid1d   HSGFNQGYNFAEAVNF
      Kdm5   HAGFNTGYNFNEAVNF
```

```
PHD Domains
   Jarid1d   CQVCSRGDEDDKLLFCDGCDDNYHIFCLLPPLPEIPRGIWRCPKCI
      Kdm5   CIVCRKTNDPKRTILCDSCDKPFHIYCLSPPLERVPSGDWICNTCI
```

```
JmjN Domains
   Jarid1d   PPECPVFEPSWAEFQDPLGYIAKIRPIAE--KSGICKIRPPADWQPPFAV
      Kdm5   MEEIPALYPTEQEFKNPIDYLSNPHIKRLGVRYGMVKVVPPNGFCPPLSI
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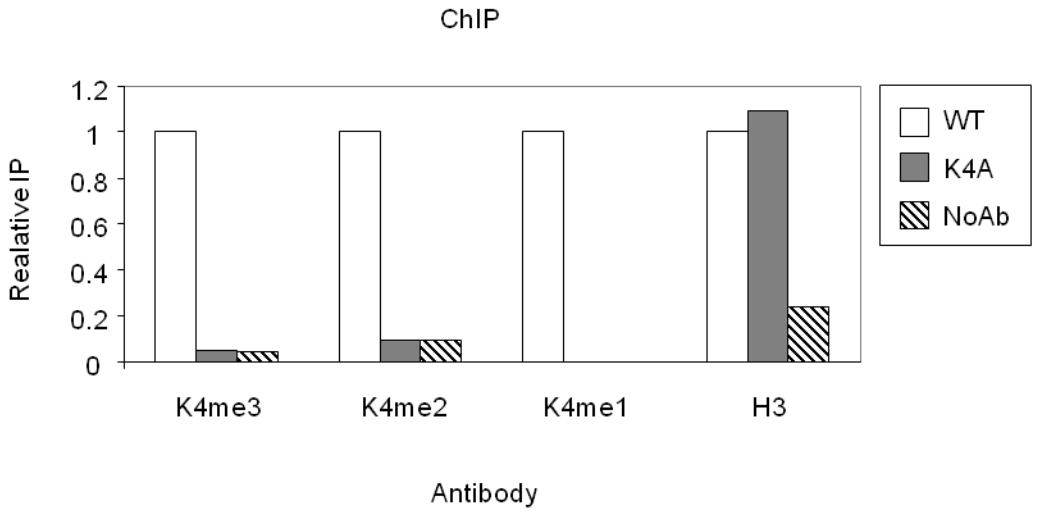
Supplementary Figure 2. Specificity of histone antibodies.



Section 4 Additional Results & Discussion

RESULTS & DISCUSSION

JHD2 Overexpression Reduces H3 K4me3 Globally In Vivo

To ask whether Kdm5 affects H3 K4 methylation *in vivo*, we engineered yeast strains in which endogenous genes encoding JmjC domain-containing proteins (including *JHD2*) were driven by a galactose-inducible *GAL1* promoter, one of yeast's strongest promoters. Yeast with endogenous *JHD2* driven by either its endogenous promoter or the *GAL1* promoter were grown in galactose to induce and whole-cell extracts were analyzed by western analysis with methyl-specific antibodies. As expected based on our *in vitro* demethylase reactions, strains in which *JHD2* was driven by the inducible promoter had lower global H3 K4me3 levels than strains in which *JHD2* was driven by its endogenous promoter (Figure 1). The effect on H3 K4me3 levels was specific since H3 K36me3 levels were not greatly reduced. As a control, total histone H3 levels were comparable between these strains.

We note that our manuscript reported that a *JHD2* deletion strain did not have expectedly increased H3 K4 methyl levels globally. The reasons are unclear however several explanations are possible. Demethylase activity with endogenous Kdm5 levels may be too low for its loss to produce a detectable global effect. Redundant demethylase activities may exist *in vivo*, although no other H3 K4-specific demethylases have been reported to date. Alternatively, Kdm5 loss might be compensated for by increased Set1 (H3 K4 methyltransferase) abundance or activity such that global H3 K4 methylation levels are unchanged.

Kdm5 demethylation mechanism

While our *in vitro* demethylase reactions with Kdm5 and core histones revealed obvious decreases in H3 K4me2, 3, they revealed only a minor decrease in H3 K4me1. This might result from Kdm5 demethylating K4me1 and simultaneously converting K4me2, 3 to K4me1. To determine whether the monomethylated state was demethylated, we repeated *in vitro* demethylase reactions with histone H3 peptides in which K4 was mono-, di-, or trimethylated. Reactions with H3 K4me1 peptides resulted in a loss of the monomethylated state. However, reactions with H3 K4me2, 3 peptides resulted in a decrease in the di- or trimethylated states and an increase in the mono-, or mono- and dimethylated states. This is indicative of Kdm5 demethylating H3 K4me2, 3 to the unmethylated state but producing H3 K4me1, 2 as intermediate states.

These results are consistent with a mechanism in which Kdm5 binds its target, removes a methyl group, and dissociates, thus removing the methyl groups one at a time. Such a distributive mechanism has been noted in other chromatin modifiers such as the H3 K79 HMT Dot1 (Frederiks, F., 2008). This is in contrast to a processive mechanism in which case Kdm5 would bind and remove all the methyl groups it intends to remove before dissociating. In this case, no intermediates would be detected.

Kdm5 in the context of others' work

Our finding that Kdm5 targets methylated H3 K4 was confirmed by other groups. We note that while we used western analyses of *in vitro* reactions, one group used *in vitro* radioactive formaldehyde release assays (Liang, G., 2007), a second used mass spectrometry of yeast-purified histones (Tu, S., 2007), a third and fourth observed that Kdm5 depletion *in vivo* lengthens H3 K4me's half-life globally via western analyses and

at a number of genes via ChIP (Radman-Livaja, M., 2010; Seward, D.J., 2007), and a fifth observed global loss of H3 K4me3 *in vivo* upon *JHD2* overexpression via western analyses (Huang, F., 2010). Kdm5's demethylase activity is thus confirmed by multiple groups and methods.

We note that the *in vitro* radioactive formaldehyde release assays detected demethylation of H3 K4me2, 3, but not H3 K4me1 (Liang, G., 2007). The reason for this difference is unclear. Their substrates were recombinant H3 histones with either K4me1 or both K4me2 and me3 which were created by HKMTs. We used calf thymus-purified histones that have many endogenous PTMs and synthetically-derived histone H3 peptides. Since histone tails are unstructured, we believe our results with unstructured peptides are valid. Reaction conditions like buffer composition or incubation time could have inhibited the others' demethylation reactions. In support of our results, it was recently reported that if H3 K4me3 is eliminated *in vivo* via a Set1/COMPASS mutation, Kdm5 overproduction then produces a slight H3 K4me1 decrease globally (Huang, F., 2010).

We noted in our manuscript that while *JHD2* deletion and overexpression altered H3 K4 methyl levels at *GAL1* during induction and repression, we failed to confirm Kdm5 recruitment here using ChIP. Since then, others have similarly reported that JmjC proteins Jhd1 and Rph1, which demethylate H3 K36, can influence H3 K36me levels inside genes but are not detectably enriched there by ChIP (Kim, T.S., 2007). This suggests that the inability to detect JmjC protein recruitment to genes that are affected by them may be a general property of the JmjC proteins rather than a failure by our ChIP methods. Their structure may simply be hard to crosslink to histones, their *in vivo*

histone interactions may be too transient to be detectable, or they may preferentially demethylate chromatin modifiers, making their effects on histones indirect *in vivo*.

METHODS

Yeast strain production and galactose-induction

GAL promoter insertions were performed as described previously (Longtine, M.S., 1998) and confirmed by PCR. Yeast were grown to mid-log phase in YP+Dextrose, then were grown in YP+Raffinose for 2 hours, then grown in YP+Galactose for 1 hour to induce *GAL1-JHD2*.

Whole-cell extract (WCE) preparation

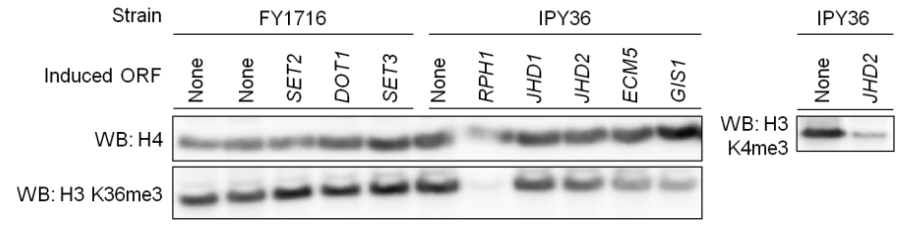
Yeast were grown in YPD (or YP+Galactose for overexpressions) to mid-log phase, resuspended in TENG-300 buffer (50mM Tris-Cl pH 7.5, 300mM NaCl, 0.5% NP-40, 1mM EDTA, 10% glycerol, 0.5mM PMSF, protease inhibitors), beat with silica beads, and sonicated after which lysates were cleared by centrifugation at 14krpm. Bradford assays determined protein concentrations.

Western analyses

Samples were run on polyacrylamide gels, transferred to PVDF, and probed with antibodies against total H4 (Millipore 05-858) or H3 K4me3 followed by incubation with chemiluminescence reagent. Signals were visualized with a Fujifilm LAS-3000 Image Reader.

Figure 1. *JHD2* overexpression reduces H3 K4me3 levels globally. Yeast with endogenous *JHD2* driven by its endogenous promoter or a *GAL1* promoter were grown in galactose after which whole-cell extracts were checked by western analyses. Induced genes and antibodies used are respectively indicated above and to the left of each blot.

Figure 1



DISSERTATION CHAPTER 3

Histone Lysine Methylation in *Saccharomyces cerevisiae*

Chapter Outline

Section 1 Chapter Summary

The work in this chapter resulted in a publication (below) confirming and characterizing monomethylation of *Saccharomyces cerevisiae* histone H4 lysine 20.

Edwards, C.R., Dang, W., and Berger, S.L. Histone H4 Lysine 20 of *Saccharomyces cerevisiae* is Monomethylated and Functions in Subtelomeric Silencing. Biochemistry. Volume 50, 2011, 10473-10483.

The work and its supplementary materials are presented in sections two and three of this chapter, respectively. References have been integrated into the dissertation bibliography. Additional results are presented in section four.

Previous studies have indicated that lysine methylations correlating with compacted or repressive chromatin, including at H3 K9, H3 K27, and H4 K20, are absent in the budding yeast *Saccharomyces cerevisiae*. A mass spectrometry report in 2007 suggested however that *S. cerevisiae* H4 K20 may be monomethylated (H4 K20me1). This modification is conserved in higher eukaryotes where it is connected to a very large number of processes, although it is generally associated with transcriptional repression and chromatin compaction. However, understanding its overall role has been challenging due to this large number of possible functions. We chose to confirm and characterize *S. cerevisiae* H4 K20me1 since this might be a chance to refute the longstanding notion that repressive methyllysines are absent in budding yeast and since this would be an opportunity to study this interesting modification using a very genetically tractable organism.

We find that H4 K20me1 is detectable on endogenous H4 by western analysis using methyl-specific antibodies, and the signal is abrogated by H4 K20 substitutions and by competition with H4 K20me1 peptides. Using chromatin immunoprecipitation we

show that H4 K20me1 levels are highest at heterochromatic locations, including subtelomeres, the silent mating type locus, and ribosomal DNA repeats, lowest within euchromatin at centromeres and promoter/5' regions of genes, and intermediate inside genes. Further, an H4 K20A substitution strongly reduced heterochromatic reporter silencing at subtelomeres and the silent mating type loci, and led to an increase in subtelomeric endogenous gene expression. The correlation between the location of H4 K20me1 and the effect of the H4 K20A substitution, suggests that this modification plays a repressive function.

H4 K20me1 levels do not show cell cycle-regulation but decrease during replicative ageing. This modification also may negatively contribute to survival during DNA damage. Our results indicate that H4 K20me1 is evolutionarily conserved from simple to complex eukaryotes and suggest that it may be the first negative regulatory histone methylation in *S. cerevisiae*.

Section 2 Histone H4 Lysine 20 of *Saccharomyces cerevisiae* is Monomethylated and Functions in Subtelomeric Silencing

ABSTRACT

Histones undergo posttranslational modifications that are linked to important biological processes. Previous studies have indicated that lysine methylation correlating with closed or repressive chromatin is absent in the budding yeast *Saccharomyces cerevisiae*, including at H4 lysine 20 (K20). Here we provide functional evidence for H4 K20 monomethylation (K20me1) in budding yeast. H4 K20me1 is detectable on endogenous H4 by western analysis using methyl-specific antibodies, and the signal is abrogated by H4 K20 substitutions and by competition with H4 K20me1 peptides. Using chromatin immunoprecipitation we show that H4 K20me1 levels are highest at heterochromatic locations, including subtelomeres, the silent mating type locus, and ribosomal DNA repeats, and lowest at centromeres within euchromatin. Further, an H4 K20A substitution strongly reduced heterochromatic reporter silencing at telomeres and the silent mating type locus, and led to an increase in subtelomeric endogenous gene expression. The correlation between the location of H4 K20me1 and the effect of the H4 K20A substitution, suggests that this modification plays a repressive function. Our findings reveal the first negative regulatory histone methylation in budding yeast, and indicate that H4 K20me1 is evolutionarily conserved from simple to complex eukaryotes.

INTRODUCTION

DNA exists within the cell wrapped around protein octamers composed of two copies each of histones H2A, H2B, H3, and H4. These histones participate in most DNA-related events such as transcription, replication, DNA repair, and chromatin compaction, and undergo numerous posttranslational modifications (PTMs) that influence these processes. Lysine methylation is one such modification and occurs on six lysines (H3 lysines 4, 9, 27, 36, and 79 and H4 lysine 20) from the fission yeast *Schizosaccharomyces pombe* to humans, the only exception being H3 K27, which is not known to be methylated in fission yeast. Lysine can be reversibly mono-, di-, or trimethylated and this modification is associated with different biological phenomena depending on the site and degree of methylation (Kouzarides, T., 2007).

H4 K20 is a particularly interesting residue since its methylation is linked to many physiological processes. The K20 methylated form recruits the methyllysine-binding protein L3MBTL1 to promote chromatin compaction (Min, J., 2007; Trojer, P., 2007; Kalakonda, N., 2008), and also recruits the respective human and fission yeast DNA repair proteins 53BP1 and Crb2 to sites of DNA damage (Sanders, S.L., 2004; Botuyan, M.V., 2006). The methyltransferase Set8 (PrSet-7) localizes to replication forks in human cells to monomethylate H4 K20; and disrupting Set8 function results in replication defects (Huen, M.S., 2008; Jørgensen, S, 2007; Tardat, M., 2007). H4 K20me1 is enriched at genes and linked to transcription, which may be associated with transcriptional attenuation (Vakoc, C.R., 2006; Congdon, L.M., 2010). In addition, in mammals, mono- and tri-methylated H4 K20 localize respectively to the transcriptionally silent X chromosome Barr body and pericentromeric heterochromatin (Schotta, G., 2004).

In addition to the role of H4 K20 methylation, the residue itself may be linked to heterochromatin function as part of a patch of basic amino acids ($K_{16}RHRK_{20}$). In budding yeast, this patch, in particular the RHR motif, recruits or regulates several chromatin proteins, including Isw2 ATP nucleosome remodeling complex, Sir2/3/4 deacetylase complex, and Dot1 methylase (Fazzio, T.G., 2005; Altaf, M., 2007; Fingerman, I.M., 2007). Lysine 20, however, has been less well studied in budding yeast, and its role and modifications have not been elucidated.

While lysine methylation associated with active transcription (H3 K4, K36, K79) is conserved from budding yeast to humans, lysine methylation associated with gene repression (H3 K9 and K27, and H4 K20), is generally thought to be absent in *S. cerevisiae* (Fang, J., 2002; Nishioka, K., 2002; Schotta, G., 2004). However, intriguingly, mass spectrometry suggested that H4 K20me1 exists in budding yeast in low abundance (Garcia, B.A., 2007). Because of the important role of H4 K20 in histone-protein interactions, and the conservation of its methylation throughout higher organisms, we sought to confirm the presence of H4 K20 methylation in *S. cerevisiae*, and to investigate possible functional roles for the modification and the K20 residue itself.

MATERIALS AND METHODS

Plasmids. *SET4* was amplified by the Expand High Fidelity PCR System (Roche), cloned into pBM272 (GAL promoter, CEN, ARS, *URA3*), and sequenced. Amino acid substitutions were engineered into the H3/H4 plasmid pRM204 (*HHT2*, *HHF2*, CEN, ARS, *TRP1*) using the QuikChange site-directed mutagenesis kit (Agilent) and confirmed by sequencing. pBY011 (GAL promoter, CEN, ARS, *URA3*) overexpression plasmids were acquired from the Yeast FLEXGene Collection (Hu, Y., 2007).

Yeast strains. Supplementary table S1 lists strains used in this study. Gene deletions and GAL promoter insertions were performed as described previously (Longtine, M.S., 1998). Plasmid transformations were performed using standard lithium acetate methods. Deletions, insertions, transformations, plasmid shufflings, and histone FLAG tags were confirmed by PCR, sequencing, and FLAG westerns as necessary.

Strains with ORFs deleted or overexpressed were created as follows. Heterozygous Diploid Deletion Collection (Winzeler, E.A., 1999) clones were sporulated to produce haploid methyltransferase deletion clones (YCE 002-016) after which mating tests and PCR confirmed ploidy and deletions respectively. To acquire additional methyltransferase deletion strains (YCE EH A2-C4), genes were deleted in the H3/H4 shuffle strain JPY12 after which JPY12 and the deletion strains were transformed with FLAG-H4 plasmids (pRM204). Strains were then grown on synthetic complete (SC) media lacking tryptophan to select for pRM204 and dilute out the original JPY12 histone plasmid. *RKM2* and demethylase deletion strains were created by standard gene knockout methods. Overexpression strains were created by transforming pBM272 or

FLEXGene Collection plasmids (pBY011) containing galactose-inducible genes into yeast or by integrating galactose-inducible promoters into the genome.

Strains with subtelomeric *URA3* and *ADE2* reporters plus wild-type or mutant histones were created as follows. pRM204 with wild-type or mutant H3/H4 genes were transformed into UCC1369 to create YCE UA1 to UA9 after which strains were grown on SC media lacking tryptophan to select for pRM204 and dilute out the original histone plasmid. Plasmids were similarly transformed into UCC7262 to make YCE UC1 to UC9, and UCC7266 to make YCE UD1 to UD9. UCC1369, UCC7262, and UCC7266 are reported elsewhere (Leeuwen, F. van, 2002).

All other mutant histone strains were created as follows. pRM204 (or derivatives of this) containing wild-type, FLAG-tagged, or mutant H3/H4 genes were transformed into the H3/H4 shuffling strain FY1716 after which SC media containing 5-FOA was used to select against the original FY1716 histone plasmid. Strains with either WT or K20 substitutions of H4 integrated into the genome were made as previously described (Dang, W., 2009).

Whole-cell extract (WCE) preparation & FLAG-affinity purification. Yeast were grown in YPD (or YP+Galactose for overexpressions) to mid-log phase, resuspended in TENG-300 buffer (50mM Tris-Cl pH 7.5, 300mM NaCl, 0.5% NP-40, 1mM EDTA, 10% glycerol, 0.5mM PMSF, protease inhibitors), beat with silica beads, and sonicated after which lysates were cleared by centrifugation at 14krpm. Bradford assays determined protein concentrations. Anti-FLAG-agarose beads (Sigma) were incubated with WCEs overnight and then washed with TENG-300. FLAG peptides (Sigma) then competed off FLAG-tagged proteins.

Western analyses. Samples were run on polyacrylamide gels, transferred to PVDF, and probed with antibodies followed by incubation with chemiluminescence reagent. Signals were visualized with a Fujifilm LAS-3000 Image Reader. Supplementary table S2 lists antibodies used in this study. Roche supplied calf thymus histone H4.

Dot blots & peptide competitions. Peptides matching the first thirty amino acids of budding yeast histone H4 plus a C-terminal cysteine were synthesized with no modifications, acetylated K16, monomethylated K20, or both modifications (Baylor College of Medicine Protein Chemistry Core Laboratory). Peptides matching the higher eukaryote histone H4 lysine 20 epitope without modifications or with mono, di, or trimethylated lysine 20 were purchased from ABCam (ab2622, ab14964, ab17043, ab17567, ab21044). Ab21044 spans residues 16-25; all others span residues 17-24. For dot blots, known amounts of peptides were spotted onto PVDF and probed with antibodies. For peptide competitions, westerns were performed as usual except that antibodies were first incubated with peptides at room-temperature for one hour.

Phenotype assays and determination of reporter expression. Yeast from YPD cultures were washed and resuspended in water, serially diluted, and spotted onto media that were then incubated at 30°C (37°C for heat sensitivity). Yeast with a *URA3* reporter were spotted onto SC media, SC media lacking uracil, or SC media with 5-FOA followed by incubation at 30°C. Yeast with an *ADE2* reporter were grown on YPD plates at 30°C and then left at 4°C for several days to allow pigment accumulation. Images were recorded with a scanner.

Replicative lifespan assay. Replicative lifespan assays and data analysis were performed as previously described (Dang, W., 2009).

Chromatin immunoprecipitation assay and determination of RNA levels. For ChIP, yeast were crosslinked with 1% formaldehyde at room temperature for 10 min followed by chromatin immunoprecipitation as described previously (Wyce, A., 2007). To extract RNA, yeast were lysed via bead-beating after which RNA was purified from extracts using the Qiagen RNeasy kit and converted to cDNA using random hexamers (IDT) and the Applied Biosystems Taqman reverse transcriptase kit. cDNAs and ChIP DNA were analyzed using the Applied Biosystems 7900HT Fast Real-Time PCR System.

RESULTS

Lysine 20 of histone H4 is monomethylated in budding yeast.

To determine whether lysine 20 of histone H4 is monomethylated in *S. cerevisiae*, we acquired a commercially available anti-H4 K20me1 polyclonal antibody (ABcam ab9051) and tested it in dot blots using $H4_{1-30}$ peptides that were unmodified, monomethylated at K20 (H4 K20me1), acetylated at K16 (H4 K16ac), or had both modifications. The antibodies preferentially recognized monomethylated versus unmethylated peptides and showed no affinity for H4 K16ac (Fig. 1A). The antibody also comparably detected H4 K20me1 in the presence or absence of H4 K16ac.

We then determined whether H4 K20me1 is detectable by western analysis of whole-cell extracts (WCEs) from a variety of strains. An epitope was detected migrating similarly to calf thymus H4 in all strains tested regardless of genetic background (S288C, SK1, and W303), mating type, ploidy, or whether the H4 gene was present in the genome or on a plasmid (Fig. 1B). Importantly, substitution of H4 K20 with the chemically similar arginine (to mimic unmodified lysine) abrogated the signal. We also probed WCEs with anti-H4 K20me2, 3 antibodies but failed to detect these modifications (Fig. S2).

To confirm that the western signal was from H4 rather than a similarly-migrating protein, we performed westerns with WCEs from strains in which all copies of H4 were either FLAG-tagged or untagged (Fig. 1C). WCEs containing untagged H4 had an H4 K20me1 western signal co-migrating with calf thymus H4, whereas WCEs with FLAG-H4 had the H4 K20me1 signal migrating slower than calf thymus H4 (Fig. 1C, left). A FLAG-H4 K20R substitution abrogated this signal confirming that the slower migrating species was FLAG-H4. These WCEs were also subjected to FLAG-affinity purification after

which elutions were analyzed by western analysis. The H4 K20me1 epitope was purified from WCEs with FLAG-H4 but not from WCEs with untagged H4 or FLAG-H4 K20R (Fig. 1C, right). These results show that the epitope is present on H4, and there is no cross-reaction with any other H3 methylation.

To further confirm the presence of the K20me1 modification, WCEs from wild-type or H4 K20R yeast were probed with anti-H4 K20me1 antibodies that had been pre-incubated without peptides (control) or with different concentrations of H4 peptides having or lacking K20me1. Control competition produced a western band with the wild-type but not mutant WCEs, and this epitope was competed in a dose-dependent manner by monomethylated but not unmodified peptides (Fig. 1D). This preferential competition was seen with WCEs from yeast of three different genetic backgrounds (Fig. 1E).

As further evidence for the existence of H4 K20me1, a second commercially-available antibody (Millipore 04-735, formerly Upstate 05-735) was tested. This antibody showed specificity in dot blots for monomethylated rather than unmethylated, dimethylated, or trimethylated H4 peptides, and detection remained constant for the double modified K16ac and K20me1 peptide (Fig. 2A, B). The antibody also detected budding yeast H4 in westerns of WCEs, co-migrating with calf thymus H4, and yeast H4 detection was abrogated by alanine, arginine, glutamine, or methionine K20 substitutions but not by an H4 K16R substitution (Fig. 2C). Further, this western signal was preferentially competed by peptides with monomethylated but not unmethylated, dimethylated, or trimethylated lysine 20 (Fig. 2D). We conclude, using a wide variety of approaches and multiple antibodies, that H4 K20me1 is present in the budding yeast *S. cerevisiae*.

Approaches to identify enzymes that modify H4 K20 in budding yeast.

To identify histone methyltransferases (HMTs) and demethylases (HDMs) for this modification, a list of candidates was produced. Since most known histone lysine methyltransferases have a SET domain, we selected the twelve *S. cerevisiae* SET domain-containing proteins (*SET1* to *SET6*, *RKM1* to *RKM4*, *CTM1*, and *EFM1*). We also selected the non-SET domain-containing HMT *DOT1* and three putative arginine methyltransferases (*HMT1*, *RMT2*, and *HSL7*). We selected the five *S. cerevisiae* Jumonji-C (JmjC) domain-containing proteins (*JHD1*, *JHD2*, *RPH1*, *GIS1*, and *ECM5*), since this domain often has histone demethylase activity, and the proposed HDM *ELP3* (Chinenov, Y., 2002; Paraskevopoulou, C., 2006). These genes were individually deleted or overexpressed and H4 K20me1 levels were checked by western analysis.

H4 K20me1 levels in deletion strains were checked by western analyses of FLAG-purified histones from strains with FLAG-tagged H4 and WCEs from all other strains (Fig. 3A, 3B, S3). As positive controls, deleting *SET1* (Fig. 3A), *SET2* (Fig. S3A), or *DOT1* (Fig. 3B, S3A, S3B) decreased H3 K4me3, H3 K36me3, or H3 K79me3 levels respectively. However, none of the deletion strains had significantly altered H4 K20me1 levels. Individual deletions of 14 additional ORFs whose protein products share homology with Dot1 (Supp. Table S3) also failed to abrogate the H4 K20me1 western signal.

Since enzyme redundancy might prevent H4 K20me1 levels from changing with individual or even double deletions, we overexpressed candidates individually and checked WCEs by western analysis (Fig. 3C, S4). As positive controls, overexpressing *RPH1* (Fig. 3C, S4B, S4C) and *JHD2* (Fig. S4B) decreased H3 K36me3 and H3 K4me3 levels respectively. However, H4 K20me1 levels did not change upon induction of any

of the candidates. In summary, deletion or overexpression of known or potential HMTs or HDMs did not alter H4 K20me1 levels in yeast.

Examination of crosstalk between H4 K20me1 and other modifications.

To test whether other histone modifications affect H4 K20me1, WCEs from strains with substitutions of known modified histone H3 and H4 residues were probed with anti-H4 K20me1 antibodies. H4 K20 substitutions eliminated the H4 K20me1 signal whereas all other substitutions (e.g. the triple substitution H4 K5R/K8R/K12R – all acetylated residues) had no effect (Fig. 2C). Substitutions of H3 K4, H3 K36, and H3 K79 were not tested since deleting or overexpressing *SET1*, *SET2*, or *DOT1* did not affect H4 K20me1 levels (Fig. 3, S3, S4).

We then tested whether, conversely, H4 K20me1 altered H4 K16ac levels. H4 K16R and H4 K16Q mutations eliminated the K16ac signal, whereas H4 K20 substitutions showed no effect on K16ac (Fig. 2C). H4 K16ac levels were also checked at various locations in the genome by ChIP and were not affected by H4 K20R substitution (data not shown). We conclude that H4 K20me1 is not altered by other abundant modifications (most of which correlate with transcription), nor does H4 K20 substitution affect H4 K16ac.

H4 K20me1 Abundance at Genomic Heterochromatin and Euchromatin

To determine the distribution of the methylation at key locations in the genome, we performed ChIP against H4 K20me1 and total H4 followed by qPCR of various locations. We checked heterochromatic locations and non-heterochromatic locations including upstream of and throughout the body of several genes as well as centromeres which,

unlike in higher eukaryotes, are not known to contain heterochromatin (Buhler, M., 2009; Ishii, K., 2009). We found that the H4-normalized ChIP signal for H4 K20me1 was higher than IgG-mock ChIPs for all locations tested, but not in the H4 K20R substitution control ChIP (Fig. 4B). We found a preferential distribution of H4 K20me1 levels within heterochromatic regions, including subtelomeres, rDNA, and the silent mating type loci *HML* (Fig. 4A, 4B, 4C lower panel). The lowest level of H4 K20me1 was found at centromeres (Fig. 4A, 4B, 4C lower panel). In general, genes showed an intermediate level between heterochromatin (highest levels) and centromeres (lowest levels) (Fig. 4A, 4B, 4C lower panel). In addition, transcribed regions/open reading frames (ORFs) of genes were higher in K20me1 compared to the upstream promoter regions and at 5' and 3' ends of genes (Fig. 4A, 4C, and 4D).

As a further control for these results, we examined ChIP signals for H3 K4me3 normalized to total histone H4. As expected, and in contrast to the distribution of H4 K20me1, H3 K4me3 levels were lower at heterochromatic locations and higher at some euchromatic locations, particularly several centromeres and the 5' region of an ORF (Fig. 4C, compare upper panel with H3 K4me3 to lower panel with H4 K20me1).

Phenotypic assays of H4 K20 substitution mutants in heterochromatic gene silencing, stress pathways, and during replicative aging.

As discussed above, H4 K20 is part of a patch of basic residues ($K_{16}RHRK_{20}$) that is linked to heterochromatin. Since we detected preferential localization of H4 K20me1 at heterochromatin, and since K20me1 in higher eukaryotes is linked to heterochromatin, chromatin compaction, and gene silencing, we investigated whether budding yeast H4 K20 and its methylation are linked to heterochromatic silencing in yeast. We used classic

heterochromatic silencing reporters at telomeres and *HML*, and also assayed subtelomeric endogenous gene expression.

Expression of a *URA3* reporter integrated at heterochromatic regions subtelomere VIIL (Fig. S5A), *HMR* (Fig. S5B), or *HML* (Fig. 5A) in yeast that have either wild-type or mutant histones was determined by comparing growth on nonselective SC plates to selective SC-URA plates (increased expression causes increased growth) or SC+5FOA plates (increased expression causes decreased growth). As positive controls, H4 K16R, H4 K16Q, and H3 K79R mutants caused silencing defects of subtelomeric *URA3*, and H4 K16R and H4 K16Q mutants caused silencing defects of *URA3* at *HMR* and *HML* loci (Fig. 5A, S5). We found that H4 K20A showed dramatic silencing defects at subtelomere VIIL, *HMR*, and *HML*, whereas H4 K20R did not change reporter expression at these tested locations. Strains with an *ADE2* reporter at subtelomere VR were also analyzed by observing yeast coloration (red and white respectively indicate silencing and expression). While H4 K16R, H4 K16Q, and H4 K20A mutations caused *ADE2* silencing defects, an H4 K20R substitution did not (Fig. 5A, S5). Importantly, H4 K20A substitution did not globally increase H4 K16ac levels in the cell (Fig. 2C) indicating that the reduction of silencing was not due to an indirect effect on acetylation.

To determine if similar derepression occurs at endogenous genes, we checked expression levels of genes proximal and distal to the telomere of chromosome VIIL. The most telomere-proximal gene is *COS12* and its location is similar to that of the subtelomeric *URA3* reporter used above (Takahasi, Y., 2011) (Fig. S5). While wild-type and H4 K20R strains had little or no detectable *COS12* expression, H4 K20A strains showed strong derepression of this gene (Fig. 5B, lower panels). Interestingly, while

these effects were seen at the telomere-proximal *COS12*, the distal *MNT2* and *ADH4* showed no such derepression with any H4 K20 substitutions, whereas numerous intermediate genes showed de-repression with the H4 K20A substitution that became less intense as the distance from the telomere increased. Similar results were seen whether mRNA levels were normalized to *ACT1* mRNA (Fig. 5B, lower panels) or 18S rRNA (data not shown). Genes *PAU11* and *YGL260w* were not included due to qPCR difficulties resulting from their homologies to other genes in the genome.

To determine whether these K20A-mediated silencing defects correlate with levels of H4 K20me1, we performed ChIP for this mark across the chromosome VIIL subtelomeric region. Interestingly, the telomere-proximal locations contained higher H4 K20me1 relative to the telomere-distal locations, and the level graded at the genes in-between (Fig. 5B, upper panel). We noted that the four most telomere-proximal genes we tested (*COS12*, *YGL262w*, *YPS5*, and *YGL258w-a*) had H4 K20A-mediated de-repression that was greater than two-fold whereas the three most telomere-distal genes we tested (*VEL1*, *MNT2*, and *ADH4*) had H4 K20A-mediated de-repression that was less than two-fold or non-existent. While the H4 K20me1 levels decreased as distance from the telomere increased, we noted that H4 K20me1 levels amongst the telomere-distal group of genes were significantly lower than amongst the telomere-proximal group of genes (Fig. 5B, upper panel). The observations that H4 K20A, but not K20R, reduced heterochromatic silencing and increased subtelomeric transcription are discussed in detail below.

We assayed H4 K20 substitution mutants under a wide variety of growth conditions to test many different gene signaling pathways. Wild-type, H4 K20A, and H4 K20R yeast grew similarly on rich and synthetic media plates at 30°C (Fig. S6), and wild-

type, H4 K20R, and H4 K20M yeast showed similar growth kinetics in liquid YPD media at 30°C (data not shown). H4 K20A and H4 K20R mutants displayed no obvious growth advantages or disadvantages compared to wild-type yeast under a variety of stress conditions (Fig. S6 and summarized in Table 1). Thus, any effects of H4 K20A do not appear to be the result of general defects in chromatin structure, stress response, or cell viability.

Acetylation of H4 K16, a residue proximal to H4 K20, increases at heterochromatin during replicative aging in budding yeast (Dang, W., 2009). Since we observed heterochromatic localization of H4 K20me1, we investigated whether H4 K20me1 levels change during replicative aging. In two yeast strains, older cells had higher H4 K16ac levels by western analysis of histones, as expected (Dang, W., 2009). Interestingly, H4 K20me1 levels were dramatically reduced during aging compared to young cells, opposite to K16ac (Fig. 5C).

DISCUSSION

While previous analyses indicated that H4 K20 is not methylated in *S. cerevisiae* (Fang, J., 2002; Nishioka, K., 2002; Schotta, G., 2004), mass spectrometry data suggested that H4 K20me1 exists in low abundance (Garcia, B.A., 2007). Using multiple methods, we demonstrate that *S. cerevisiae* H4 K20 is, indeed, monomethylated. Anti-H4 K20me1 antibodies detected an H4 epitope in western blots of WCEs from various strains and purified H4. These western signals from endogenous histones were abrogated by a K20R substitution and were preferentially competed by H4 K20me1 peptides (Fig. 1 & 2). In contrast, we detected neither di- (consistent with previous reports (Fang, J., 2002; Nishioka, K., 2002)) nor tri-methylated H4 K20 in budding yeast (Fig. S2). Since

antibodies are central to modification studies, we note that there was significant lot-to-lot variation in the efficacy of H4 K20me1 antibodies, typical of many modification antibodies (Egelhofer, T.A., 2011).

Our evidence supporting the existence of budding yeast H4 K20me1 is not entirely surprising. First, studies reporting its absence according to western analysis actually used antibodies raised against H4 K20me2 peptides, and not K20me1 (Fang, J., 2002; Nishioka, K., 2002). Second, although budding yeast lack orthologues to higher eukaryote H4 K20 methylase enzymes, chromatin modifying enzymes sometimes lack expected protein sequence homologies or domains. For example, most HMTs have a SET domain and yet the H3 K79 HMT Dot1 has a different catalytic domain (Nguyen, A.T., 2011). Third, although the amino acid sequence surrounding budding yeast H4 K20 is not totally conserved in higher eukaryotes, it is fully conserved in fission yeast (Supp. Fig. S1), which has K20 methylation (Sanders, S.L., 2004).

Functions of H4 K20 monomethylation.

H4 K20me1 is reported in higher eukaryotes to be associated with transcriptional silencing, chromatin compaction, and heterochromatin. We therefore hypothesized that this modification would preferentially associate with heterochromatic regions of the budding yeast genome. Levels of H4 K20me1 were significantly higher at heterochromatic locations and lower at euchromatic locations, including centromeres which, unlike in higher eukaryotes, are not heterochromatic (Fig. 4A, 4C lower panel) (Buhler, M., 2009; Ishii, K., 2009). We also found H4 K20me1 to be higher in the middle of genes relative to upstream regions and 5’ or 3’ ends (Fig. 4A, 4D), consistent with findings from mammalian studies showing enrichment of H4 K20me1 inside of genes

(Vakoc, C.R., 2006; Congdon, L.M., 2010). H4 K20me1 may have a compaction function within genes, similar to H3 K36me3, which localizes to the 3' half of genes and recruits HDACs to promote chromatin compaction (Carrozza, M.J., 2005).

The previous relationship of H4 K20 methylation to heterochromatin, DNA replication, DNA repair, and chromatin compaction in higher eukaryotes, prompted testing of these pathways in budding yeast. It is intriguing that H4 K20A substitution produced dramatic silencing defects at subtelomeres, including the most telomere-proximal gene *COS12* and multiple adjacent subtelomeric genes (Fig. 5B lower panel), and at the silent mating type loci *HMR* and *HML* (Fig. S5B, 5A). Interestingly, H4 K20me1 levels showed a striking correlation with K20A-mediated silencing defects along regions proximal and distal to telomere VIIL (Fig. 5B, compare lower and upper panels). The silencing defects are not an indirect effect of increasing acetylation at H4 K16, because global H4 K16ac levels were comparable in strains bearing wild type H4 relative to K20A (Fig. 2C), and was not due to general disruption of chromatin structure since the H4 K20A mutant did not show increased transcription at all genes examined (Fig. 5B lower panels). Previous studies of the $K_{16}RHRK_{20}$ patch *in vitro* and *in vivo* have not identified a role for K20 in recruitment or regulation of heterochromatic or other complexes, such as Sir2/3/4, Isw2, or Dot1 (Fazzio, T.G., 2005; Altaf, M., 2007; Fingerman, I.M., 2007). It will be interesting in future studies to determine the role of K20me1 in promoting repression at subtelomeres and at the silent mating type loci.

In contrast to K20A, the H4 K20R substitution did not show silencing defects at subtelomeres and silent mating type loci, nor did K20R exhibit phenotypes associated with DNA replication inhibitors, DNA damaging agents, or other stress conditions (Fig. 5A, S5, S6 and Table 1). Nonetheless we favor the view that there is a function for H4

K20me1, based on the strong correlation between the location of the modification at heterochromatic regions, and the highly specific defect of H4 K20A in telomere-proximal regulation. One possible explanation for the absence of an effect of K20R in silencing, or other processes, is that effector proteins may interact with this basic patch through binding to both the residues and K20me1, such that interactions are compromised by loss of either, but only sufficiently to produce a detectable phenotype with loss of the residues. In this view, K20A may alter the structure sufficiently to disrupt binding, but loss of methylation alone via K20R, would not disrupt binding.

An alternative possibility is that the methylation is redundant with other pathways. Such a scenario is not uncommon in budding yeast chromatin. Indeed, it is remarkable that certain well-known modifications, such as H2B K123 ubiquitylation, which is present on more than half the histone H2B proteins in the cell, has only a modest phenotype when abrogated by a K123R substitution. Yet, when K123R is combined with deletion of *GCN5*, the major H3 acetyltransferase (whose deletion also results in only minor reduction of transcription), there is a strong synthetic effect, causing very low transcription (Kao, C.F., 2004).

Since acetylation of the adjacent H4 K16 participates in replicative aging (Dang, W., 2009), we examined whether H4 K20me1 is also linked to aging. Interestingly, H4 K20me1 levels were reduced in older compared to younger yeast (Fig. 5C). This lowered detection is not due to H4 K16ac obscuring the K20me1 signal, because substituting K16 to arginine or glutamine does not globally alter K20me1 levels (Fig. 2C), nor does K16ac prevent K20me1 recognition in peptide dot blots (Fig. 1A, 2A). This result is consistent with association of H4 K20me1 and H4 K16ac with opposing chromatin states, i.e. the former with condensed and silent chromatin, whereas the latter

with open and active chromatin. It is possible that the decrease in K20me1 in old cells might affect protein recruitment or chromatin structure. We note that we have not observed a change in lifespan in an H4 K20R substitution (data not shown), thus a role of H4 K20me1 during aging is unclear. However, we do not believe that such a function would occur by altering H4 K16ac levels, since levels of this adjacent modification are not altered by H4 K20R substitutions (Fig. 2C).

Unexpected histone modifications and unidentified enzymes in budding yeast. Budding yeast histones have additional lysines methylations, some of which are conserved in higher eukaryotes. Recently H2B K37 methylation was identified at low levels in *S. cerevisiae*, and this residue and methylation are both conserved in complex eukaryotes; substitution mutations here (K37R and K37A) have no detectable phenotype however (Gardner, K.E., 2011). Similarly, H3 K42 methylation was recently identified in budding yeast and may be linked to transcription; lysine to arginine substitution mutation here however also has no detectable phenotype *(*Hyland, E.M., 2012*)*. These findings, along with our data on H4 K20 and its methylation, indicate that histone lysine methylation, including low abundance marks, may be more common than previously appreciated, and may have pathway-specific functions that are difficult to reveal.

Furthermore, similar to H4 K20me1, individual deletions of *DOT1* and all known SET domain-containing genes do not abrogate H2B K37 or H3 K42 methylation *(*Gardner, K.E., 2011; Hyland, E.M., 2012*)* and combinatorial deletions of three known arginine methyltransferases do not abrogate H3 R2 methylation *(*Kirmizis, A., **YEAR***)*. While enzyme redundancies may be the reason in some cases, unidentified methyltransferases may instead be responsible for these previously unknown budding

yeast modifications. Genome-wide deletion and overexpression screens and biochemical methods may help to discover the responsible enzymes.

While H4 K20 and H2B K37 are conserved and methylated from *S. cerevisiae* to higher eukaryotes, H3 K9 and H3 K27 are also conserved and methylated in higher organisms, but reportedly unmethylated in *S. cerevisiae*. Our western analyses have not revealed H3 K9me in budding yeast (data not shown), however our search for this mark has not been extensive; we note that recent mass spectrometry data detected low levels of this modification (Garcia, B.A., 2007), and the *S. cerevisiae* HDM Rph1 targets H3 K9me *in vitro* (Klose, R.J., 2007). Whether any other “absent” marks are actually conserved in this model organism remains to be determined.

ACKNOWLEDGEMENTS

We thank Aaron Gitler for the FLEXGene Collection overexpression plasmids, Dan Gottschling for the *URA3*/*ADE2* reporter strains, Edel Hyland for deletion strains containing FLAG-tagged histone H4, Kristin Ingvarsdottir for strains with deleted or overexpressed demethylases, Edward Winter for pBM272, and Kajia Cao for assistance with statistical methods.

Table 1. Stress conditions tested.[1]

Conditions tested with H4 K20R	Conditions tested with H4 K20A
YPD or SC at 37°C (Heat sensitivity)	YPD or SC at 37°C (Heat sensitivity)
YP + Galactose (Galactose utilization)	YP + Galactose (Galactose utilization)
YP + Acetate (Acetate utilization)	YP + Acetate (Acetate utilization)
YP + Ethanol & glycerol (Ethanol & glycerol utilization)	YP + Ethanol & glycerol (Ethanol & glycerol utilization)
YP + Raffinose (Raffinose utilization)	YP + Sucrose (Sucrose utilization)
YPD + 100-125mM Hydroxyurea (DNA replication stress)	YPD + 100-125mM Hydroxyurea (DNA replication stress)
YPD + 10-15µg/mL Camptothecin (Topoisomerase inhibition stress)	YPD + 10-15µg/mL Camptothecin (Topoisomerase inhibition stress)
YPD + 1M NaCl (Osmolarity stress)	YPD + 1M NaCl (Osmolarity stress)
SC + UV light (DNA damage)	SC + UV light (DNA damage)
YPD + 0.01-0.05% MMS (DNA damage)	
YPD + 0.02% H_2O_2 (Oxidative stress)	
SC Lacking inositol (Inositol utilization)	
YP + Low glucose (respiration)	

[1]Stress conditions used in phenotype assays. Growth of wild-type and H4 K20 substitution yeast were compared between stress and non-stress conditions.

Figure 1. H4 K20me1 detectable in budding yeast. (A) Peptides matching the first 30 amino acids of H4 with or without acetylated lysine 16 or monomethylated lysine 20 were spotted onto PVDF and probed with antibodies. Peptides, peptide amounts, and antibodies are respectively indicated to the left, top, and bottom of the blots. (B) H4 K20me1 is detectable in whole-cell extracts (WCEs) from strains of different genetic backgrounds, mating types, and ploidies, is detectable whether H4 genes are present in the genome or on a plasmid, and is abrogated by an H4 K20R substitution. WCEs were analyzed on polyacrylamide gels, transferred to PVDF, and probed with antibodies. H4 levels are a loading control. Calf thymus H4 is a positive antibody control. Antibodies are indicated to the left of each blot. Strains, genetic backgrounds, and mating types are indicated above each lane. Strains FY1716, YKI071, and YWD193 have H3/H4 genes present only on a plasmid. (C) To confirm that the H4 K20me1 signal is H4 and not a similarly migrating protein, WCEs with untagged H4, FLAG-H4, or FLAG-H4 K20R were subjected to FLAG-affinity purification. WCEs and elutions were analyzed by western analyses. Antibodies are indicated to the left of each blot. The FLAG tag and K20R substitution are indicated above each lane. An asterisk indicates a non-specific band that obscures FLAG-H4 in WCEs. Arrows indicate untagged H4 in WCEs and FLAG-H4 in eluates. (D & E) H4 K20me1 western signals preferentially competed by monomethylated rather than unmethylated H4 peptides. WCEs were analyzed on polyacrylamide gels, transferred to PVDF, and probed with anti-H4 K20me1 antibodies (1mg/mL) that were pre-incubated with no peptides or H4 peptides (100ng/mL or 1ng/mL in D, 100ng/mL in E) that were or were not monomethylated at lysine 20. H4 levels are a loading control. Antibodies are indicated to the left of each blot. Strains, peptides, and

peptide concentrations are indicated above each blot. Arrow indicates H4 K20me1. Asterisk indicates a non-specific band.

Figure 2. H4 K20me1 detectable with Millipore antibody 04-735. (A, B) Peptide dot blots as in figure 1A. (C) Substitutions of modifiable histone residues do not affect H4 K20me1 levels and H4 K20 substitutions do not affect H4 K16ac levels. WCEs from yeast with wild-type or mutant histones were analyzed by western analyses. Antibodies and histone mutations are respectively indicated to the left of and above each blot. Asterisk indicates a non-specific band present on blot probed for H4 K16ac. (D) Western blots with peptide competitions as in 1D, E.

Figure 3. Deleting and overexpressing candidate genes does not change H4 K20me1 levels. (A, B) FLAG-H4 was purified by FLAG-affinity purification from strains with candidate HMTs deleted. WCEs and FLAG elutions were analyzed by western analysis. Antibodies and strains are respectively indicated to the left of and above each blot. FLAG levels are a loading control. (C) WCEs from strains with candidate HMTs overexpressed from a plasmid were analyzed by western analysis. Antibodies and strains are respectively indicated to the left of and above each blot. H4 levels are a loading control. An H4 K20R strain serves as a negative antibody control.

Figure 4. ChIP-qPCR analysis of H4 K20me1. Sonicated chromatin from yeast with WT or K20R histone H4 were immunoprecipitated by antibodies against H4 K20me1 or total H4 and co-purified DNA was analyzed by qPCR. Bars indicate H4 K20me1 levels normalized to total H4 levels (A) or no antibody or H4 K20me1 levels normalized to input

levels (B). Genomic locations are indicated below each pair of bars. (C) Comparison of H3 K4me3 versus H4 K20me1 levels relative to H4 levels at several loci. (D) H4 K20me1 levels relative to H4 levels across several regions: *ACT1* ORF, *RCK2-YEF3* region, and *YLR454w* region. Horizontal bars and arrows underneath graph represent genome and genes respectively. Position of bar graph bars above genes indicates locations of checked regions. A, B, and D represent means and standard error of the mean (S.E.M.) of three experiments.

Figure 5. Phenotype analysis of H4 K20me1. (A) Dilutions of *hml::URA3 ADE2*-TEL-VR yeast with wild-type or mutant histones were grown on synthetic complete (SC), SC-URA, or SC+5FOA media. Media and strains are indicated above and to the right of each plate respectively. Increased growth on SC-URA media and decreased growth on SC+5FOA media compared to SC media indicate increased *URA3* expression. White and red yeast coloration respectively indicates *ADE2* expression and silencing. (B) H4 K20me1 levels at and expression of genes proximal and distal to telomere 7L were determined by ChIP of H4 K20me1 and total H4 and by qPCR of harvested RNAs. (top) ChIP was as in Figure 4. H4 K20me1 levels per total H4 levels are shown with means and standard errors of the mean. ChIP locations residing along chromosome 7L are indicated in the map by black triangles while above numbers indicate which locations correspond to which bars in the above graph. Locations 2-5 were compared to locations 6-8 using a Wilcoxon rank sum test and generated the indicated P-value. (bottom) RNA was extracted from yeast with WT, K20R, or K20A histone H4. mRNA levels of genes proximal and distal to telomere 7L were determined by qPCR and normalized to *ACT1* mRNA. Means and standard error of the means are shown. WT and H4 K20A samples

were compared using a T-test and generated the indicated P-values. (C) H4 K20me1 levels are lower in replicatively older than younger yeast. WCEs from replicatively young or old yeast were analyzed by western analyses. Antibodies are indicated to the left of each blot. Samples and strains are indicated above each lane. Average numbers of bud scars (cell divisions) for each yeast sample are indicated in parentheses. H3 levels are a loading control.

Figure 1

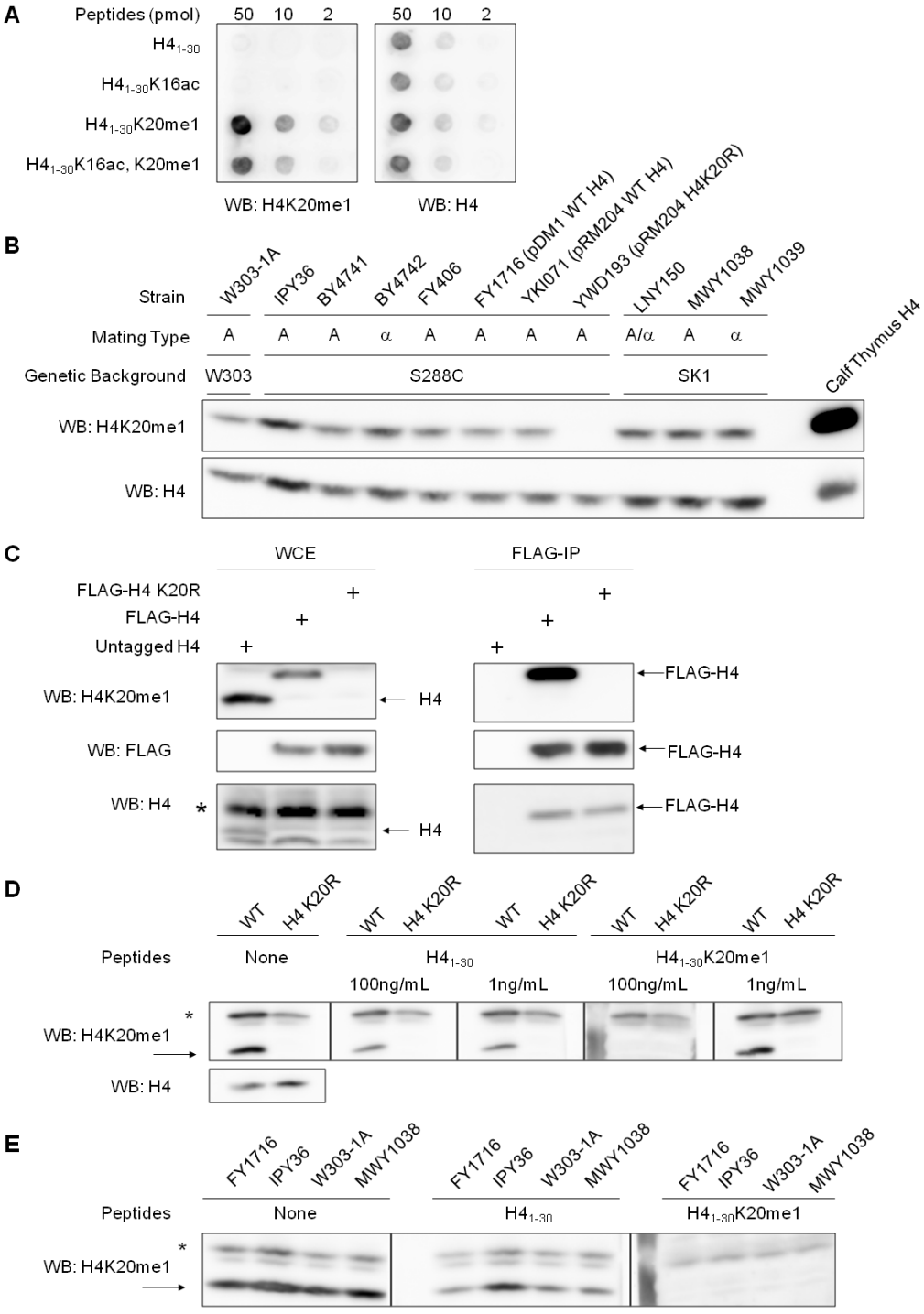

A
Peptides (pmol)
50 10 2
50 10 2
H4 1-30
H4 1-30 K16ac
H4 1-30 K20me1
H4 1-30 K16ac, K20me1
WB: H4K20me1
WB: H4
B
Strain
W303-1A
IPY36
BY4741
BY4742
FY406
FY1716 (pDM1 WT H4)
YKI071 (pRM204 WT H4)
YWD193 (pRM204 H4K20R)
LNY150
MWY1038
MWY1039
Calf Thymus H4
Mating Type
A A A α A A A A A/α A α
Genetic Background
W303
S288C
SK1
WB: H4K20me1
WB: H4
C
WCE
FLAG-IP
FLAG-H4 K20R
FLAG-H4
Untagged H4
WB: H4K20me1
WB: FLAG
WB: H4
H4
FLAG-H4
D
WT
H4 K20R
Peptides
None
H4 1-30
H4 1-30 K20me1
100ng/mL
1ng/mL
WB: H4K20me1
WB: H4
E
FY1716
IPY36
W303-1A
MWY1038
Peptides
None
H4 1-30
H4 1-30 K20me1
WB: H4K20me1

Figure 2

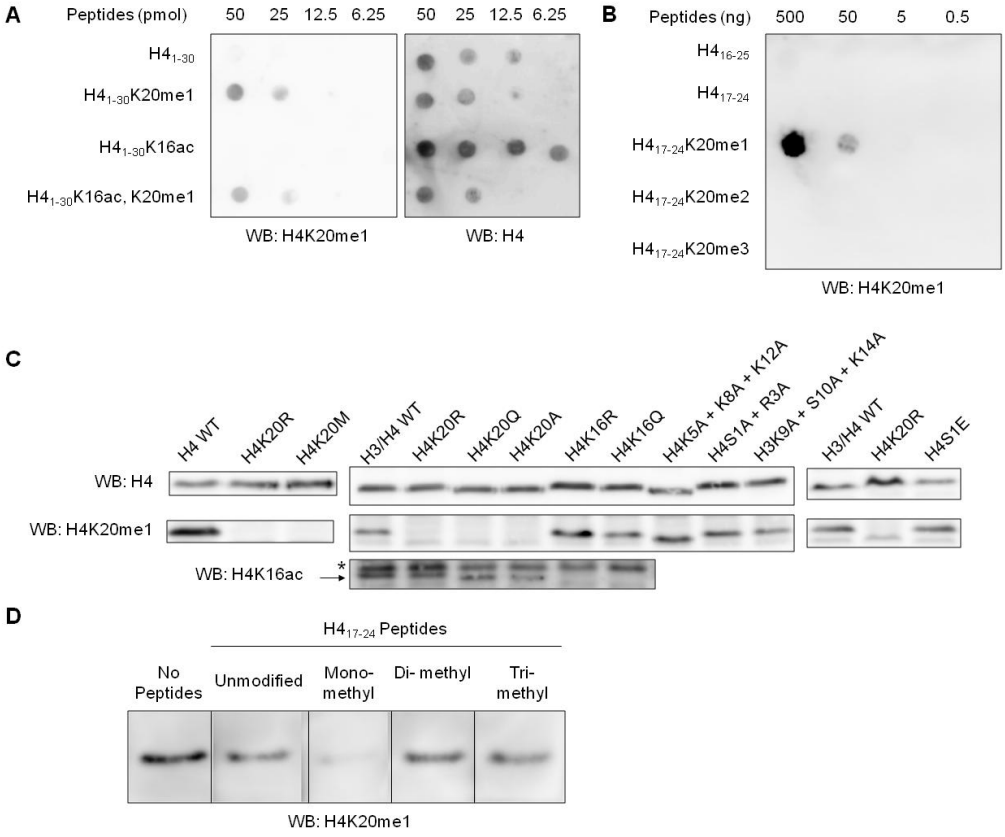

A
Peptides (pmol)
50 25 12.5 6.25 50 25 12.5 6.25
H4 1-30
H4 1-30 K20me1
H4 1-30 K16ac
H4 1-30 K16ac, K20me1
WB: H4K20me1
WB: H4
B
Peptides (ng)
500 50 5 0.5
H4 16-25
H4 17-24
H4 17-24 K20me1
H4 17-24 K20me2
H4 17-24 K20me3
WB: H4K20me1
C
H4 WT
H4K20R
H4K20M
H3/H4 WT
H4K20R
H4K20Q
H4K20A
H4K16R
H4K16Q
H4K5A + K8A + K12A
H4S1A + R3A
H3K9A + S10A + K14A
H3/H4 WT
H4K20R
H4S1E
WB: H4
WB: H4K20me1
WB: H4K16ac
*
D
H4 17-24 Peptides
No Peptides
Unmodified
Mono-methyl
Di- methyl
Tri-methyl
WB: H4K20me1

Figure 3

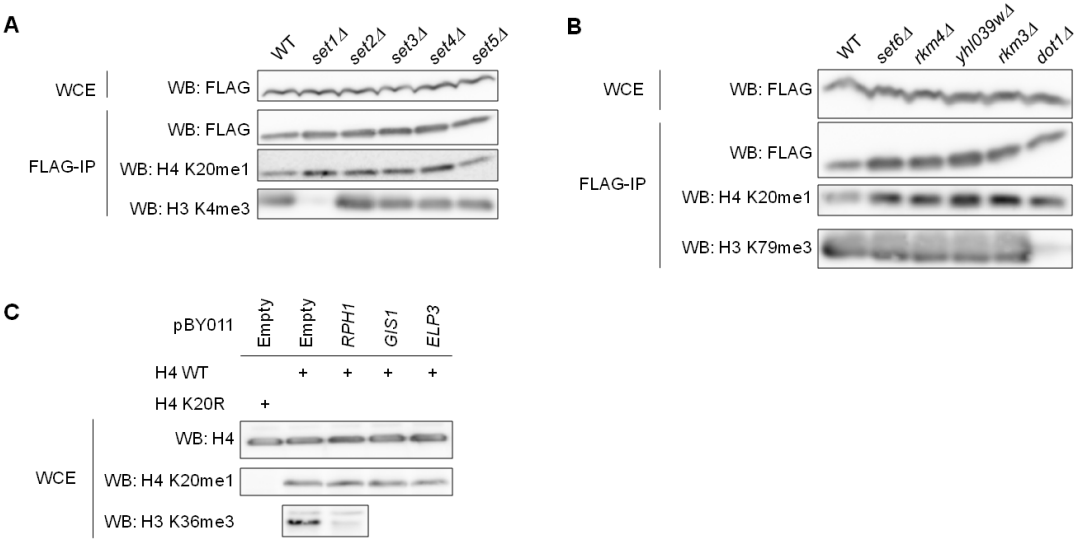

A
WT
set1Δ
set2Δ
set3Δ
set4Δ
set5Δ
WCE
WB: FLAG
FLAG-IP
WB: FLAG
WB: H4 K20me1
WB: H3 K4me3
B
WT
set6Δ
rkm4Δ
yhl039wΔ
rkm3Δ
dot1Δ
WCE
WB: FLAG
FLAG-IP
WB: FLAG
WB: H4 K20me1
WB: H3 K79me3
C
pBY011
Empty
Empty
RPH1
GIS1
ELP3
H4 WT
+
+
+
+
H4 K20R
+
WCE
WB: H4
WB: H4 K20me1
WB: H3 K36me3

Figure 4

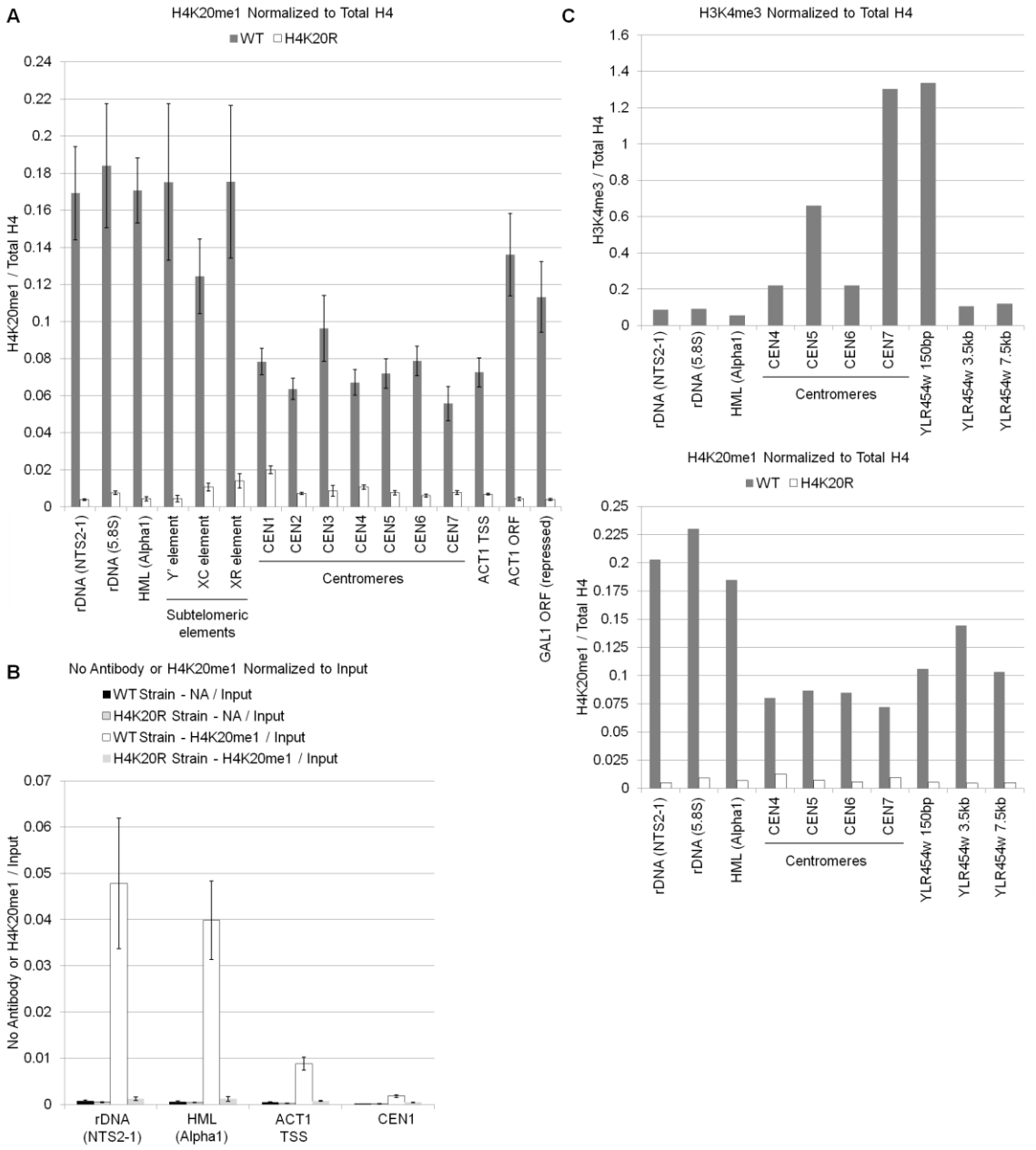

A
H4K20me1 Normalized to Total H4
WT
H4K20R
H4K20me1 / Total H4
rDNA (NTS2-1)
rDNA (5.8S)
HML (Alpha1)
Y' element
XC element
XR element
Subtelomeric elements
CEN1
CEN2
CEN3
CEN4
CEN5
CEN6
CEN7
Centromeres
ACT1 TSS
ACT1 ORF
GAL1 ORF (repressed)
B
No Antibody or H4K20me1 Normalized to Input
WT Strain - NA / Input
H4K20R Strain - NA / Input
WT Strain - H4K20me1 / Input
H4K20R Strain - H4K20me1 / Input
No Antibody or H4K20me1 / Input
rDNA (NTS2-1)
HML (Alpha1)
ACT1 TSS
CEN1
C
H3K4me3 Normalized to Total H4
H3K4me3 / Total H4
CEN4
CEN5
CEN6
CEN7
YLR454w 150bp
YLR454w 3.5kb
YLR454w 7.5kb
H4K20me1 Normalized to Total H4
WT
H4K20R
H4K20me1 / Total H4

Figure 4

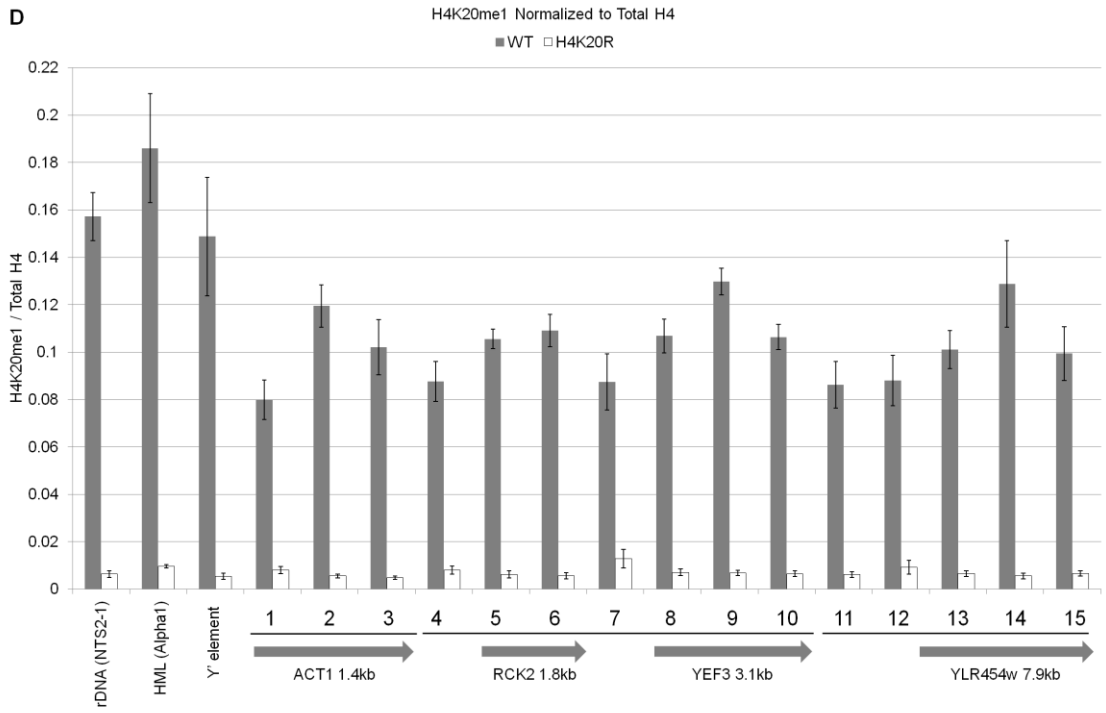

D
H4K20me1 Normalized to Total H4
WT
H4K20R
H4K20me1 / Total H4
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
rDNA (NTS2-1)
HML (Alpha1)
Y' element
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
ACT1 1.4kb
RCK2 1.8kb
YEF3 3.1kb
YLR454w 7.9kb

Figure 5

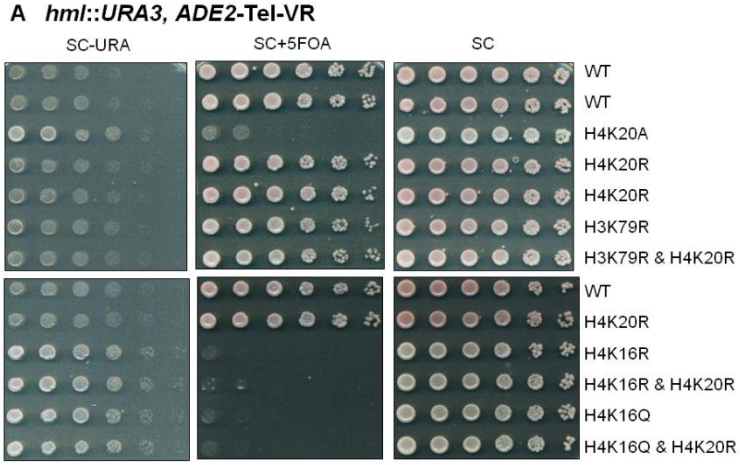

A hml::URA3, ADE2-Tel-VR
SC-URA
SC+5FOA
SC
WT
WT
H4K20A
H4K20R
H4K20R
H3K79R
H3K79R & H4K20R
WT
H4K20R
H4K16R
H4K16R & H4K20R
H4K16Q
H4K16Q & H4K20R

Figure 5

B

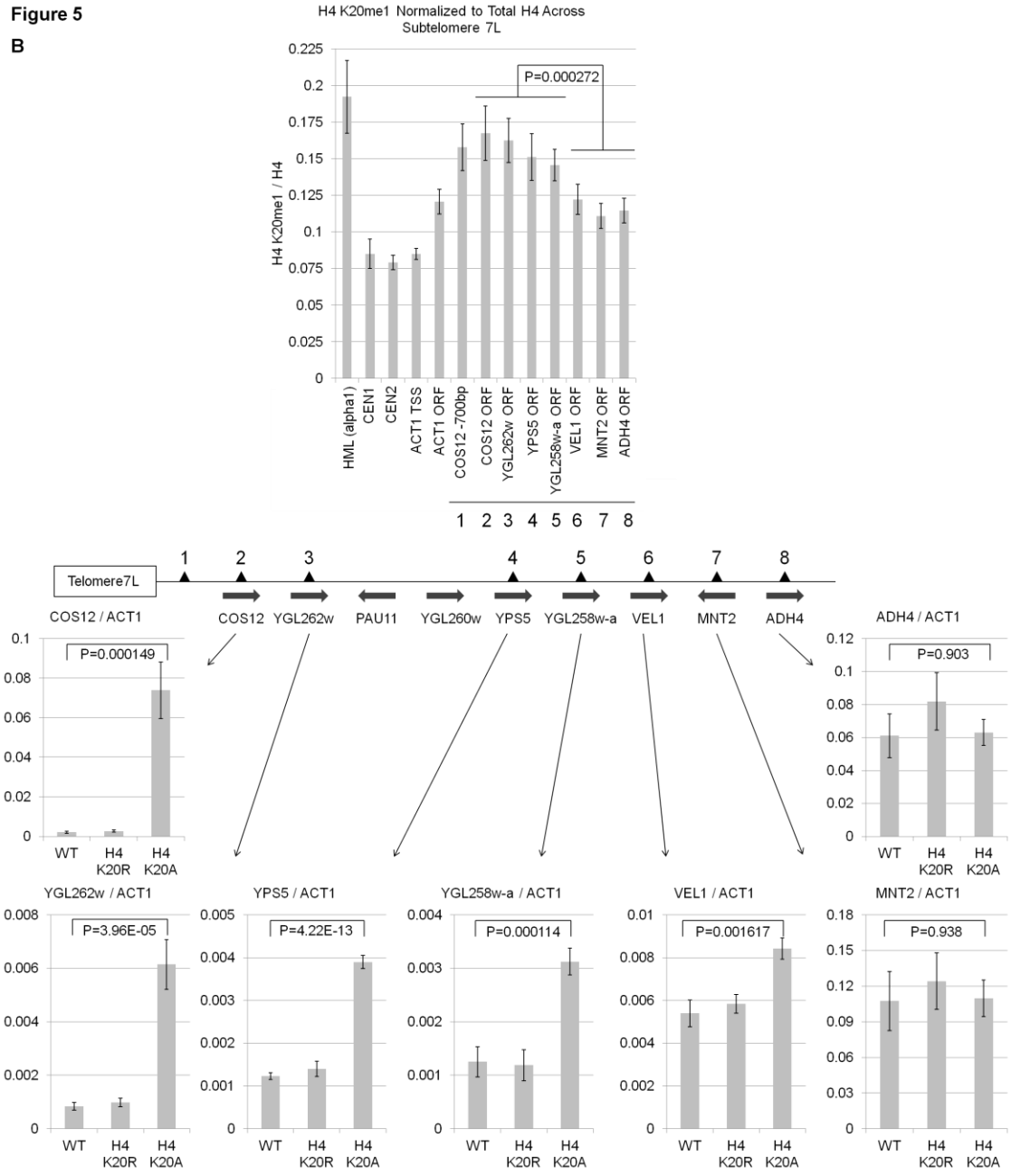
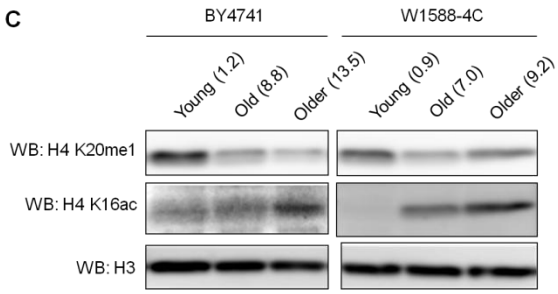

H4 K20me1 Normalized to Total H4 Across Subtelomere 7L
H4 K20me1 / H4
P=0.000272
HML (alpha1)
CEN1
CEN2
ACT1 TSS
ACT1 ORF
COS12 -700bp
COS12 ORF
YGL262w ORF
YPS5 ORF
YGL258w-a ORF
VEL1 ORF
MNT2 ORF
ADH4 ORF
1 2 3 4 5 6 7 8
Telomere7L
COS12
YGL262w
PAU11
YGL260w
YPS5
YGL258w-a
VEL1
MNT2
ADH4
COS12 / ACT1
P=0.000149
ADH4 / ACT1
P=0.903
YGL262w / ACT1
P=3.96E-05
YPS5 / ACT1
P=4.22E-13
YGL258w-a / ACT1
P=0.000114
VEL1 / ACT1
P=0.001617
MNT2 / ACT1
P=0.938
WT
H4 K20R
H4 K20A

Figure 5


C
BY4741
W1588-4C
Young (1.2)
Old (8.8)
Older (13.5)
Young (0.9)
Old (7.0)
Older (9.2)
WB: H4 K20me1
WB: H4 K16ac
WB: H3

Section 3 Supplementary Materials for Section Two

Supplementary Table S1. Yeast strains used in this study.

Figure 1	Genotype	Source
W303-1A	Mata ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15	Thomas, B.J., 1989
IPY36 (Isogenic to SB301)	Mata his3Δ200 leu2Δ1 ura3-52 trp1ΔhisG	Sterner, D.E., 2002
BY4741	Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ0	Brachmann, C.B., 1998
BY4742	Mata his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0	Brachmann, C.B., 1998
FY406	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hta1-htb1)::LEU2 (hta2-htb2)::TRP1 pSAB6(HTA1, HTB1, CEN, URA3)	Hirschhorn, J.N., 1995
FY1716	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pDM1(HHT2, HHF2, CEN, URA3)	Nathan, D., 2006
YKI071	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pRM204(HHT2, HHF2, CEN, TRP1)	This study
YWD193	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pWD93(pRM204 HHT2, hhf2 K20R, TRP1, CEN)	This study
LNY150	Mata/Mata leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG lys2-SK1/lys2-SK1 his4-N/his4-G ura3-SK1/ura3-SK1 ho::LYS2/ho::LYS2	L. Neigeborn
MWY1038	Mata leu2::hisG trp1::hisG lys2-SK1 his4-N/G ura3-SK1 ho::LYS2	Edward Winter
MWY1039	Mata leu2::hisG trp1::hisG lys2-SK1 his4-N/G ura3-SK1 ho::LYS2	Edward Winter
YKI156	Mata his3D200 leu2D1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study

YCE017	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE003(pRM204 HHT2, FLAG-hhf2 K20R, CEN, TRP1)	This study
YKI071	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pRM204(HHT2, HHF2, CEN, TRP1)	This study
YWD193	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pWD93(pRM204 HHT2, hhf2 K20R, TRP1, CEN)	This study
Figure 2	Genotype	Source
YWD 1000	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2	Dang, W., 2009
YWD 1120	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 hhf2 K20R	This study
YWD 1121	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 hht2 A15V-hhf2 K20M	This study
YCE074	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1)	This study
YCE075	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE007(pRM204 HHT2, hhf2 K20R, CEN, TRP1)	This study
YCE129	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE028(pRM204 HHT2, hhf2-K20Q, CEN, TRP1)	This study
YCE126	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE005(pRM204 HHT2, hhf2-K20A, CEN, TRP1)	This study
YWD123	Mata his3D200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pWD23(pRM204 HHT2, hhf2 K16R, CEN, TRP1)	Dang, W., 2009
YWD125	Mata his3D200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pWD25(pRM204 HHT2, hhf2 K16Q, CEN, TRP1)	Dang, W., 2009
YKI076	Mata his3D200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pRM204(HHT2, hhf2 K5A K8A K12A, CEN, TRP1)	This study

TY99	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pTK59(pRM204 HHT2, hhf2 S1A R3A, CEN, TRP)	This study
T7	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pRM204(hht2 K9A S10A K14A, HHF2, CEN, TRP1)	This study
TY102	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pTK57(pRM204 HHT2, hhf2 S1E, CEN, TRP)	Cheung, W.L., 2005
Figure 3, S3, S4	Genotype	Source
IPY36 (Isogenic to SB301)	Mata his3Δ200 leu2Δ1 ura3-52 trp1ΔhisG	Sterner, D.E., 2002
YKI112	Mata his3Δ200 leu2Δ1 ura3-52 trp1ΔhisG rph1::KanMX	This study
YKI113	Mata his3Δ200 leu2Δ1 ura3-52 trp1ΔhisG jhd1::KanMX	Ingvarsdottir, K., 2007
YKI114	Mata his3Δ200 leu2Δ1 ura3-52 trp1ΔhisG ecm5::KanMX	This study
YKI115	Mata his3Δ200 leu2Δ1 ura3-52 trp1ΔhisG gis1::KanMX	This study
YKI127	Mata his3Δ200 leu2Δ1 ura3-52 trp1ΔhisG jhd2::HIS3	Ingvarsdottir, K., 2007
YKI188	Mata his3Δ200 leu2Δ1 ura3-52 trp1ΔhisG elp3::HIS3	This study
BY4742	Mata his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0	Brachmann, C.B., 1998
YCE 002	Mat alpha set2::KanMX his3Δ1 leu2Δ0 ura3Δ0 [met15Δ0 and/or lys2Δ0]	This study
YCE 004	Mat alpha set3::KanMX his3Δ1 leu2Δ0 ura3Δ0 [met15Δ0 and/or lys2Δ0]	This study
YCE 006	Mat alpha set4::KanMX his3Δ1 leu2Δ0 ura3Δ0 [met15Δ0 and/or lys2Δ0]	This study
YCE 008	Mat alpha set5::KanMX his3Δ1 leu2Δ0 ura3Δ0 [met15Δ0 and/or lys2Δ0]	This study
YCE 010	Mat alpha ctm1::KanMX his3Δ1 leu2Δ0 ura3Δ0 [met15Δ0 and/or lys2Δ0]	This study

YCE 012	Mat alpha rkm1::KanMX his3Δ1 leu2Δ0 ura3Δ0 [met15Δ0 and/or lys2Δ0]	This study
YCE 014	Mat alpha rkm3::KanMX his3Δ1 leu2Δ0 ura3Δ0 [met15Δ0 and/or lys2Δ0]	This study
YCE 016	Mat alpha dot1::KanMX his3Δ1 leu2Δ0 ura3Δ0 [met15Δ0 and/or lys2Δ0]	This study
YCE EH A2	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study
YCE EH A4	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 set1::KanMX pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study
YCE EH A6	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 set2::KanMX pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study
YCE EH A8	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 set3::KanMX pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study
YCE EH A10	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 set4::KanMX pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study
YCE EH A12	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 set5::KanMX pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study
YCE EH B2	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 set6::KanMX pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study
YCE EH B4	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 rkm4::KanMX pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study

YCE EH B6	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 efm1::KanMX pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study
YCE EH B8	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 rkm3::KanMX pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study
YCE EH B10	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 dot1::KanMX pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study
YCE EH B12	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 hmt1::KanMX pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study
YCE EH C2	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 rmt2::KanMX pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study
YCE EH C4	Mata his3Δ200 leu2Δ1 lys2Δ0 met15Δ0 ura3-167 trp1Δ63 ade2::his (hht1-hhf1)::natMX (hht2-hhf2)::hygMX RDN1::mURA3/HIS3 RDN1::Ty1-MET15 TELV::ADE2 hsl7::KanMX pRM204(HHT2, FLAG-HHF2, CEN, TRP1)	This study
YKI071	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pRM204(HHT2, HHF2, CEN, TRP1)	This study
YWD193	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pWD93(pRM204 HHT2, hhf2 K20R, CEN, TRP1)	This study
YCE019	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) rkm2::KanMX	This study
YCE082	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE007(pRM204 HHT2,	This study

	hhf2 K20R, CEN, TRP1) pBY011(GALprom, Gateway, CEN, URA3)	
YCE083	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, Gateway, CEN, URA3)	This study
YCE084	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, SET1, CEN, URA3)	This study
YCE085	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, SET5, CEN, URA3)	This study
YCE086	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, SET6, CEN, URA3)	This study
YCE087	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, RKM4, CEN, URA3)	This study
YCE088	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, CTM1, CEN, URA3)	This study
YCE089	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, RKM1, CEN, URA3)	This study
YCE090	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, RKM2, CEN, URA3)	This study
YCE091	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, RKM3, CEN, URA3)	This study
YCE092	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2,	This study

	HHF2, CEN, TRP1) pBY011(GALprom, EFM1, CEN, URA3)	
YCE093	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, HMT1, CEN, URA3)	This study
YCE094	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, RMT2, CEN, URA3)	This study
YCE095	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, HSL7, CEN, URA3)	This study
YCE096	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, JHD1, CEN, URA3)	This study
YCE097	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, RPH1, CEN, URA3)	This study
YCE098	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, GIS1, CEN, URA3)	This study
YCE099	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBY011(GALprom, ELP3, CEN, URA3)	This study
YCE074	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1)	This study
YCE075	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE007(pRM204 HHT2, hhf2 K20R, CEN, TRP1)	This study
YCE102	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 KanMX6:pGAL-SET2 pCE001(pRM204 HHT2, HHF2, CEN, TRP1)	This study

YCE103	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 KanMX6:pGAL-DOT1 pCE001(pRM204 HHT2, HHF2, CEN, TRP1)	This study
YCE104	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 KanMX6:pGAL-SET3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1)	This study
YKI161	Mata his3D200 leu2D1 ura3-52 trp1DhisG KanMX:pGAL-RPH1	This study
YKI163	Mata his3D200 leu2D1 ura3-52 trp1DhisG KanMX:pGAL-JHD1	This study
YKI165	Mata his3D200 leu2D1 ura3-52 trp1DhisG KanMX:pGAL-JHD2	This study
YKI167	Mata his3D200 leu2D1 ura3-52 trp1DhisG KanMX:pGAL-ECM5	This study
YKI169	Mata his3D200 leu2D1 ura3-52 trp1DhisG KanMX:pGAL-GIS1	This study
YCE123	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pBM272-Empty	This study
YCE124	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pCE029(pBM272 pGAL, SET4, CEN, URA3)	This study
YCE125	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1) pCE030(pBM272 pGAL, SET4, CEN, URA3)	This study
Fig. 4	Genotype	Source
YWD 1000	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2	Dang, W., 2009
YWD 1120	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 hhf2 K20R	This study
Figure 5A, S5	Genotype	Source
YCE UD1	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hml::URA3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1)	This study

YCE UD2	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hml::URA3 pCE005(pRM204 HHT2, hhf2 K20A, CEN, TRP1)	This study
YCE UD3	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hml::URA3 pCE007(pRM204 HHT2, hhf2 K20R, CEN, TRP1)	This study
YCE UD4	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hml::URA3 pCE014(pRM204 hht2 K79R, HHF2, CEN, TRP1)	This study
YCE UD5	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hml::URA3 pCE016(pRM204 hht2 K79R, hhf2 K20R, CEN, TRP1)	This study
YCE UD6	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hml::URA3 pCE018(pRM204 HHT2, hhf2 K16R, CEN, TRP1)	This study
YCE UD7	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hml::URA3 pCE019(pRM204 HHT2, hhf2 K16R K20R, CEN, TRP1)	This study
YCE UD8	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hml::URA3 pCE020(pRM204 HHT2, hhf2 K16Q, CEN, TRP1)	This study
YCE UD9	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hml::URA3 pCE021(pRM204 HHT2, hhf2 K16Q K20R, CEN, TRP1)	This study
YCE UA1	MATa ade2::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL-VIIL ADE2-TEL-VR hhf2-hht2::MET15 hhf1-hht1::LEU2 pCE001(pRM204 HHT2, HHF2, CEN, TRP1)	This study
YCE UA2	MATa ade2::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL-VIIL ADE2-TEL-VR hhf2-hht2::MET15 hhf1-hht1::LEU2 pCE005(pRM204 HHT2, hhf2 K20A, CEN, TRP1)	This study

YCE UA3	MATa ade2::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL-VIIL ADE2-TEL-VR hhf2-hht2::MET15 hhf1-hht1::LEU2 pCE007(pRM204 HHT2, hhf2 K20R, CEN, TRP1)	This study
YCE UA4	MATa ade2::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL-VIIL ADE2-TEL-VR hhf2-hht2::MET15 hhf1-hht1::LEU2 pCE014(pRM204 hht2 K79R, HHF2, CEN, TRP1)	This study
YCE UA5	MATa ade2::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL-VIIL ADE2-TEL-VR hhf2-hht2::MET15 hhf1-hht1::LEU2 pCE016(pRM204 hht2 K79R, hhf2 K20R, CEN, TRP1)	This study
YCE UA6	MATa ade2::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL-VIIL ADE2-TEL-VR hhf2-hht2::MET15 hhf1-hht1::LEU2 pCE018(pRM204 HHT2, hhf2 K16R, CEN, TRP1)	This study
YCE UA7	MATa ade2::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL-VIIL ADE2-TEL-VR hhf2-hht2::MET15 hhf1-hht1::LEU2 pCE019(pRM204 HHT2, hhf2 K16R K20R, CEN, TRP1)	This study
YCE UA8	MATa ade2::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL-VIIL ADE2-TEL-VR hhf2-hht2::MET15 hhf1-hht1::LEU2 pCE020(pRM204 HHT2, hhf2 K16Q, CEN, TRP1)	This study
YCE UA9	MATa ade2::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL-VIIL ADE2-TEL-VR hhf2-hht2::MET15 hhf1-hht1::LEU2 pCE021(pRM204 HHT2, hhf2 K16Q K20R, CEN, TRP1)	This study
YCE UC1	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hmr::URA3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1)	This study
YCE UC2	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hmr::URA3 pCE005(pRM204 HHT2, hhf2 K20A, CEN, TRP1)	This study
YCE UC3	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hmr::URA3 pCE007(pRM204 HHT2, hhf2 K20R, CEN, TRP1)	This study

YCE UC4	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hmr::URA3 pCE014(pRM204 hht2 K79R, HHF2, CEN, TRP1)	This study
YCE UC5	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hmr::URA3 pCE016(pRM204 hht2 K79R, hhf2 K20R, CEN, TRP1)	This study
YCE UC6	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hmr::URA3 pCE018(pRM204 HHT2, hhf2 K16R, CEN, TRP1)	This study
YCE UC7	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hmr::URA3 pCE019(pRM204 HHT2, hhf2 K16R K20R, CEN, TRP1)	This study
YCE UC8	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hmr::URA3 pCE020(pRM204 HHT2, hhf2 K16Q, CEN, TRP1)	This study
YCE UC9	MATa ade2 his3 trp1 leu2 lys2 ura3 met15 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hmr::URA3 pCE021(pRM204 HHT2, hhf2 K16Q K20R, CEN, TRP1)	This study
Figure 5B	Genotype	Source
YWD 1000	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2	Dang, W., 2009
YWD 1120	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 hhf2 K20R	This study
YWD 1119	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 hhf2 K20A	This study
Figure 5C	Genotype	Source
BY4741	Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ0	Brachmann, C.B., 1998
W1588-4C	Mata RAD5 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15	Thomas, B.J., 1989
Figure S2	Genotype	Source

FY1716	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pDM1(HHT2, HHF2, CEN, URA3)	Nathan, D., 2006
YWD193	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pWD93(pRM204 HHT2, hhf2 K20R, TRP1, CEN)	This study
YWD 1000	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2	Dang, W., 2009
YWD 1120	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 hhf2 K20R	This study
Figure S6	Genotype	Source
YWD 1000	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2	Dang, W., 2009
YWD 1120	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 hhf2 K20R	This study
YWD 1119	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 hhf2 K20A	This study
IPY36 (Isogenic to SB301)	Mata his3Δ200 leu2Δ1 ura3-52 trp1ΔhisG	Sterner, D.E., 2002
TY44	Mata his3Δ200 leu2Δ1 ura3-52 trp1ΔhisG snf2::TRP1	Lo, W.S., 2001
YCE074	Mata his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE001(pRM204 HHT2, HHF2, CEN, TRP1)	This study
YWD 156	Mata his3D200 leu2Δ1 ura3-52 trp1Δ63 lys2-128d (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pRM204(hht2 K56R, HHF2, CEN, TRP1)	Dang, W., 2009
YCE 365	Mata his3D200 leu2D1 ura3-52 trp1D63 lys2-128d (hht1-hhf1)::LEU2 *rad9*::KanMX6	This study

Supplementary Table S2. Antibodies used in this study. Millipore 04-735 was formerly Upstate 05-735.

Specificity	Company	Catalog No.	Notes
H4 K20me1	ABCAM	ab9051	Rabbit polyclonal
H4 K20me1	Millipore	04-735	Rabbit monoclonal
H4 K20me2	ABCAM	ab9052	Rabbit polyclonal
H4 K20me2	Millipore	07-747	Rabbit polyclonal
H4 K20me3	ABCAM	ab9053	Rabbit polyclonal
H4	ABCAM	ab31827	Mouse monoclonal
H4	Upstate / Millipore	05-858	Rabbit monoclonal
H3	ABCAM	ab1791	Rabbit polyclonal
FLAG	Sigma	A8592	HRP-conjugated mouse monoclonal
H3 K4me3	ABCAM	ab8580	Rabbit polyclonal
H3 K4me3	Upstate / Millipore	05-473	Rabbit polyclonal - No longer available
H3 K4me3	Active Motif	39159	Rabbit polyclonal
H3 K36me3	ABCAM	ab9051	Rabbit polyclonal
H3 K79me3	ABCAM	ab2621	Rabbit polyclonal
H4 K16 acetyl	Active Motif	39167	Rabbit polyclonal
Rabbit IgG	Bio-Rad	170-6515	HRP-conjugated goat monoclonal
Mouse IgG	Bio-Rad	170-6516	HRP-conjugated goat monoclonal

Supplementary Table S3. Additional genes whose deletions do not abrogate H4 K20me1.

Systematic Name	Standard Name
YBR141c	-
YBR225w	-
YBR261c	TAE1
YOR239w	ABP140
YMR053c	STB2
YMR209c	-
YNL022c	-
YNL092w	-
YBR271w	-
YDR083w	RRP8
YHR209w	CRG1
YIL064w	SEE1
YJR129c	-
YLR455w	-

Supplementary Figure S1. Alignment of histone H4 N-terminal sequences (residues 1-29) from several model organisms. Lysine 20 is present in boldface.

Supplementary Figure S2. H4 K20me2, 3 not detected by western analysis. Whole-cell extracts (WCEs) from yeast with WT or K20R histone H4 were analyzed by polyacrylamide gels, transferred to PVDF, and probed with antibodies. Calf thymus histone H4 serves as a positive control. Strains and antibodies used are indicated above and to the right of each blot.

Supplementary Figure S3. Deleting candidate genes individually does not change H4 K20me1 levels. (A, C, D) WCEs were analyzed by western analysis. (B) FLAG-H4 was purified by FLAG-affinity purification after which WCEs and elutions were analyzed by western analysis. Antibodies and strains are respectively indicated to the left of and above each blot. FLAG or H4 levels are a loading control.

Supplementary Figure S4. Overexpressing candidate genes individually does not change H4 K20me1 levels. (A) Candidate enzymes on plasmid pBY011 were overexpressed using a galactose-inducible promoter. (B) Candidate enzymes were overexpressed by driving the endogenous genes with a galactose-inducible promoter. (C) Candidate *SET4* on plasmid pBM272 was overexpressed using a galactose-inducible promoter. Endogenous *RPH1* or *JHD2* were overexpressed as a positive control using a galactose-inducible promoter. WCEs were analyzed by western analysis. Antibodies and strains are respectively indicated to the left of and above each blot. H4 levels are a loading control.

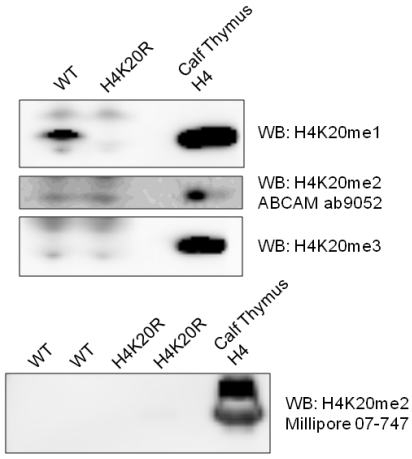
Supplementary Figure S5. Analysis of reporter expression in yeast with *URA3*-TEL-VIIL *ADE2*-TEL-VR (A) or *URA3*::*hmr ADE2*-TEL-VR (B). Dilutions of yeast with wild-type or mutant histones were grown on synthetic complete (SC), SC-URA, or SC+5FOA media. Media and strains are indicated above and to the right of each plate respectively. Increased growth on SC-URA media and decreased growth on SC+5FOA media compared to SC media indicate increased *URA3* expression. White and red yeast coloration respectively indicates *ADE2* expression and silencing.

Supplementary Figure S6. Dilutions of WT or mutant yeast were grown under non-stress or various stress-inducing conditions. Conditions and strains are indicated above and to the left of each plate respectively. H3 K56R, *snf2*Δ, and *rad9*Δ strains are positive controls for phenotypes.

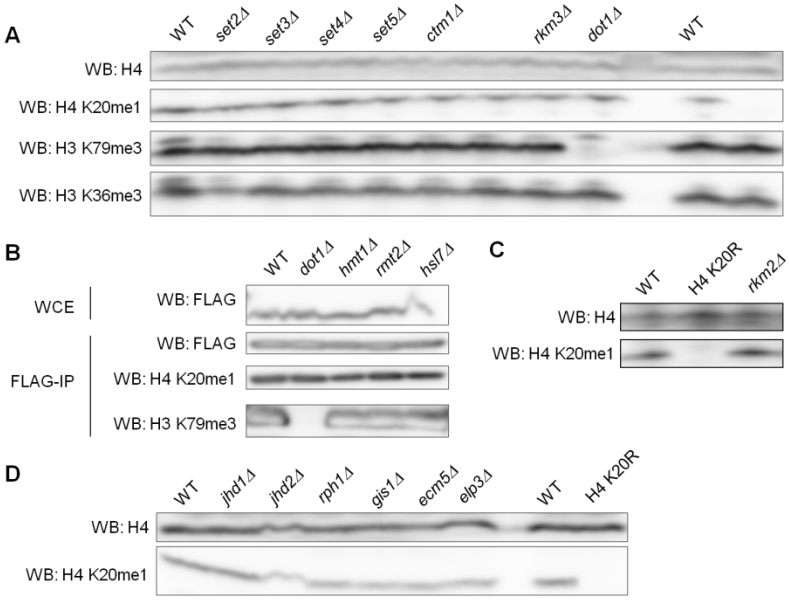
Supplementary Figure S1

Human	H4	SGRGKGGKGLGKGGAKRHR**K**VLRDNIQGI
Mouse	H4	SGRGKGGKGLGKGGAKRHR**K**VLRDNIQGI
Fly	H4	TGRGKGGKGLGKGGAKRHR**K**VLRDNIQGI
S. pombe	H4	SGRGKGGKGLGKGGAKRHR**K**ILRDNIQGI
S. cerevisiae	H4	SGRGKGGKGLGKGGAKRHR**K**ILRDNIQGI

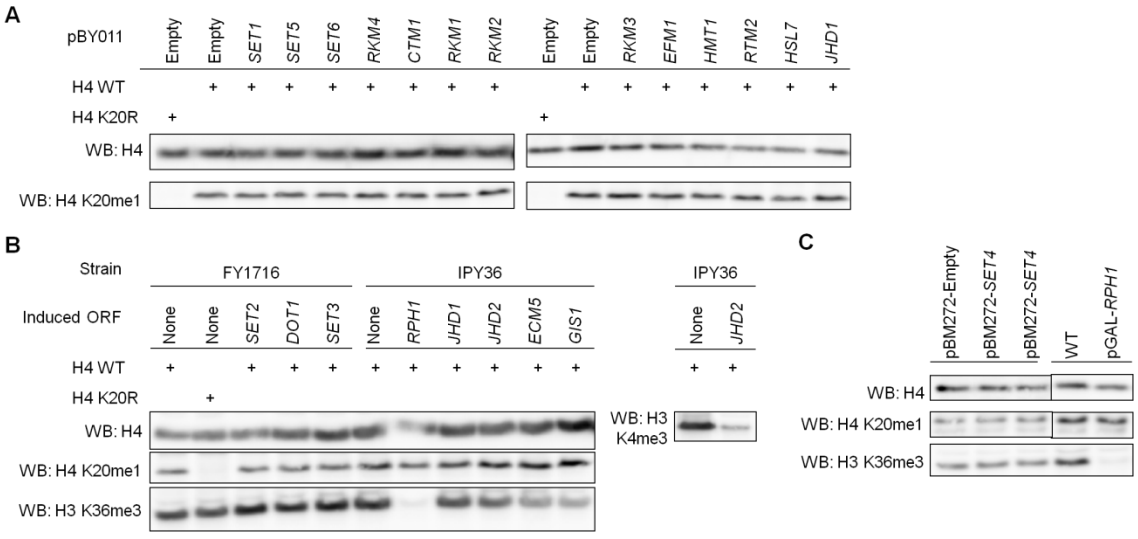
Supplementary Figure S2



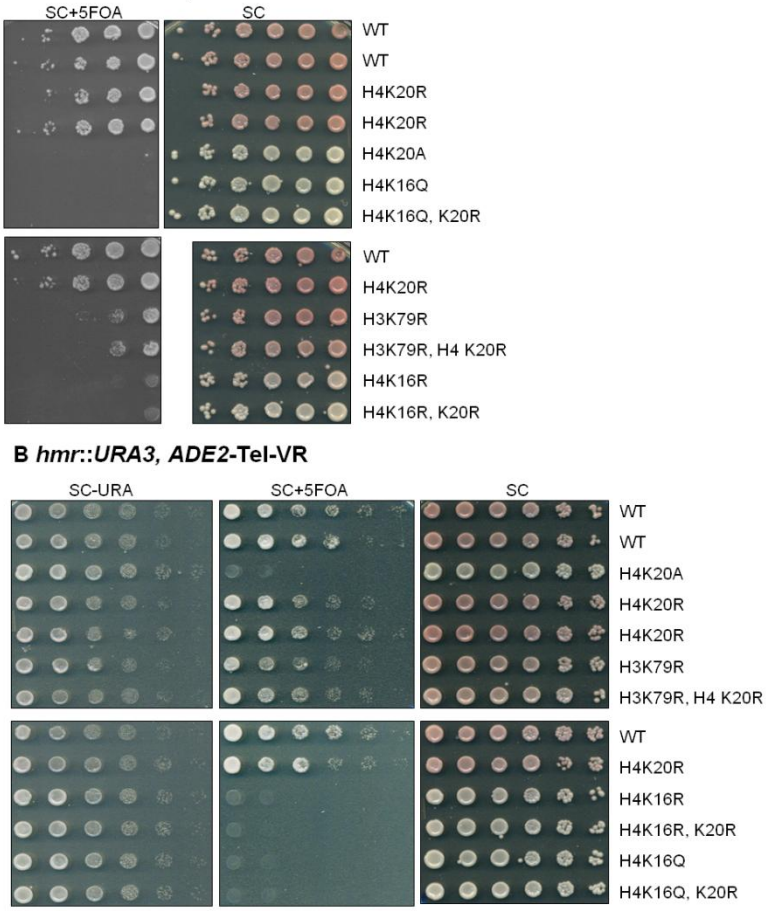
Supplementary Figure S3



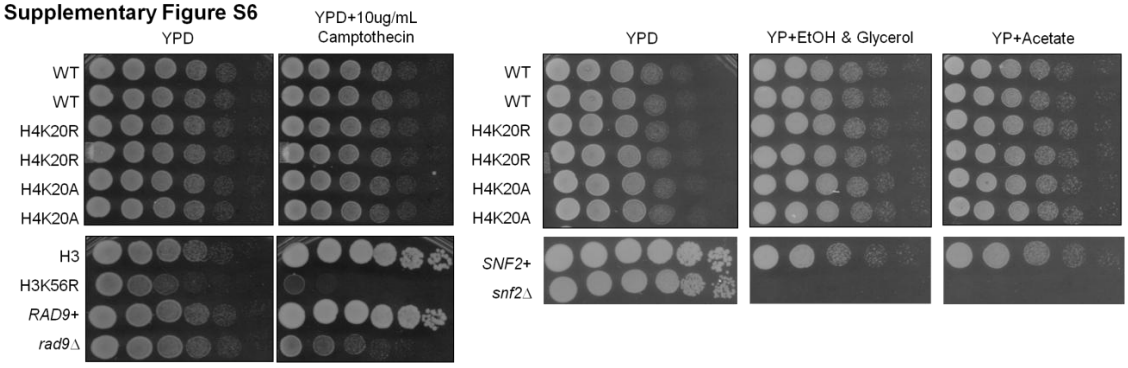
Supplementary Figure S4



Supplementary Figure S5



Supplementary Figure S6



Section 4 Additional Results

H4 K20ME1 AND THE CELL CYCLE

H4 K20me1 abundance is cell cycle-regulated in mammals

Mammalian H4 K20me1 and its HMT Set8 are thought to participate in the cell cycle; their abundance is lowest in S phase, becomes highest in G2-M phases, and decreases upon return to G1 phase. We note that some reports failed to observe any cell cycle pattern (Tardat, M., 2010; Karachentsev, D., 2007) and low abundance during S phase is counterintuitive considering reports of Set8 interactions with PCNA during replication (Huen, M.S., 2008; Jørgensen, S., 2007; Tardat, M., 2007). Many reports however using mass spectrometry, western analyses, and fluorescently-labelled overexpressed Set8, agree on the cell cycle abundance pattern (Oda, H., 2010; Wu, S., 2010; Abbas, T., 2010; Pesavento, J.J., 2008; Brustel, J., 2011). This pattern results from Set8 degradation and H4 K20me1 demethylation during S phase by the CLR4(ctd2) ubiquitin ligase and PHF8 demethylase, and by Set8 degradation during late M phase by the APC/C ubiquitin ligase (Centore, R.C., 2010; Abbas, T., 2010; Liu, W., 2010; Wu, S., 2010). A recent model proposes that this pattern restricts its different functions to the right times (Brustel, J., 2011). For example, H4 K20me1 reportedly promotes replication origin licensing (Tardat, M., 2010). Low levels at S phase and high levels at G2-M phases would thus restrict licensing to after replication. Likewise H4 K20me1 reportedly represses some replication-related genes (Abbas, T., 2010; Liu, W., 2010). Low levels at S phase would thus derepress these genes during DNA replication. Set8 and H4 K20me1 thus play important roles in the mammalian cell cycle. However, many details remain to be discovered and whether Set8's methylation of histones is the actual

mechanism is difficult to know. We thus asked whether *S. cerevisiae* H4 K20me1 is similarly cell cycle-regulated.

No evidence for H4 K20me1 variation during the S. cerevisiae cell cycle

To determine whether *S. cerevisiae* H4 K20me1 levels change during the cell cycle, we blocked yeast in G1 phase with Alpha Factor, G2-M phases with Nocodazole, and S phase with Hydroxyurea and checked modification levels by western analysis (Fig. 1). Cdc28 was similarly abundant in the different phases as expected whereas Clb2, a G2-M marker, was most abundant with Nocodazole treatment and undetectable with Alpha Factor treatment. WCEs were then loaded into protein gels to achieve similar total H4 levels and H4 K20me1 levels were checked relative to this. Unexpectedly, H4 K20me1 levels normalized to total H4 levels did not reproducibly show convincing cell cycle patterns. Similar results were seen with an Alpha Factor block and release (data not shown) in which cells were released from a G1 block and H4 K20me1 levels were checked periodically for at least one cell cycle. H4 K20me1's cell cycle regulation thus may not be conserved in *S. cerevisiae*. Finding the responsible enzyme will be helpful in confirming this by observing its cell cycle pattern. To ask whether H4 K20me1 regulates cell cycle progression, we will determine whether H4 K20R substitutions cause cell cycle progression delays.

H4 K20ME1 AND DNA DAMAGE

Mammalian H4 K20me1 and the DNA damage response

There is evidence that mammalian H4 K20me1 may function in the DNA damage response, although this role has not been extensively studied. Mouse embryonic stem

cells in culture lacking Set8 have increased DNA damage. While this may result from replication defects, it may also occur independently of this (H2AXphos foci do not generally colocalize with EdU foci and do occur in both EdU-positive and negative cells) (Oda, H., 2009). Further, Set8, H4 K20me1, and 53BP1 (a DNA damage reponse protein) foci colocalize at laser-induced DNA damage sites. Set8 and H4 K20me1 are necessary for and precede 53BP1 recruitment and importantly, SILAC experiments reported that 53BP1 in mammalian cells preferentially bound H4 K20me1 rather than H4 K20me2 peptides (Oda, H., 2010). While H4 K20me2 is generally considered the preferential *in vitro* target of 53BP1's tandem tudor domains (Botuyan, M.V., 2006), H4 K20me1 thus may also directly recruit 53BP1 *in vivo*. H4 K20me1's role in the DNA damage response is not well-studied however and whether H4 is Set8's main or only target in this process is unknown. We note that *S. pombe* H4 K20R substitutions and deletions of its HMT *SET9* abrogate H4 K20me1-3 and Crb2 recruitment (53BP1 ortholog) and cause DNA damage sensitivity, but the *in vivo* roles of each methyl state are unknown (Sanders, S.L., 2004). We therefore asked whether *S. cerevisiae* H4 K20me1 is linked to the DNA damage response.

Synthetic effects with H4 K20R substitutions

We asked whether *S. cerevisiae* H4 K20me1 loss affects survival during DNA damage. Wild-type and H4 K20 substitution strains grew similarly with DNA damage-inducing agents (Fig. 2A, B, and the manuscript). Since functional redundancies can prevent individual mutations from revealing phenotypes, we checked whether H4 K20R substitutions combined with substitutions of histone residues linked to the DNA damage response would reveal phenotypes. H3 K79 methylation is implicated in the DNA

damage response (Nguyen, A.T., 2011) however, neither UV light, Camptothecin, nor Hydroxyurea caused growth differences between H3 K79R and H4 K20R double substitution strains relative to wild-type or single substitution strains (Fig. 2B).

Rtt109 acetylates newly synthesized H3 on K56 during S phase and this is needed for survival during DNA damage (Masumoto, H., 2005; Han, J., 2007). We therefore asked whether an H3 K56R and H4 K20R double substitution would reveal a DNA damage phenotype. As reported, H3 K56R yeast grew poorly compared to wild-type yeast with Camptothecin treatment but not with vehicle alone (Fig. 2A). H4 K20R substitutions caused no growth changes compared to wild-type yeast with either Camptothecin or vehicle alone (Fig. 2A-C). Interestingly, while the combinatorial substitution and H3 K56R strains grew similarly with vehicle alone, the combinatorial strains grew moderately (~5-fold) better than the H3 K56R strains with Camptothecin treatment (Fig. 2C). The H4 K20R substitution thus moderately rescues the H3 K56R-mediated Camptothecin-sensitivity.

A Possible Role in Survival During DNA Damage

It is intriguing that the H4 K20R and H3 K56R substitutions demonstrated a synthetic effect (Fig. 2C) since this argues that H4 K20me1 may participate in the DNA damage response in *S. cerevisiae*. It is more intriguing however that the synthetic effect was a moderate rescue of the H3 K56R-mediated DNA damage sensitivity. Since H3 K56ac promotes survival upon DNA damage, these results suggest that H4 K20me1 negatively contributes to this process, opposing H3 K56ac's function. That a synthetic effect was not revealed with H3 K79R and H4 K20R combinatorial substitutions (Fig. 2B) argues that this was not simply due to having multiple mutations present.

The combinatorial H3 K56R and H4 K20R substitutions have only been tested extensively with Camptothecin. Camptothecin is a topoisomerase poison that binds to type 1 topoisomerases such that they cleave a single DNA strand but fail to ligate it. These single-stranded nicks may be converted to double-stranded DNA breaks during replication by translocating replication forks (Li, T.K., 2001). The H4 K20R substitution thus moderately rescues H3 K56R-mediated sensitivity to, most likely, double-stranded DNA breaks that occur during S phase. Whether H4 K20me1 also participates in repair of bases damage by UV light or alkylating agents, or double-stranded breaks that are detected outside of S phase, remains to be seen. In addition, since topoisomerases could affect other processes that are dependent on DNA topology, we cannot rule out that the H4 K20R and H3 K56R synthetic effects with Camptothecin treatment are linked to these other pathways. We are inclined to think however that DNA repair during S phase is the relevant pathway since H3 K56ac is closely linked to this process.

Future studies of H4 K20me1 during DNA damage induction

To further examine H4 K20me1's link to DNA damage, we will ask if a combinatorial H4 K20R and *rtt109* deletion strain has a similar synthetic effect and if synthetic effects also occur with other DNA damaging agents. This will indicate what types of damage repair H4 K20me1 is linked to and whether topoisomerases' role in double-stranded DNA break creation is the mechanism. To investigate the mechanism of H4 K20me1's contribution, we will ask whether H4 K20me1 levels change globally or locally upon DNA damage induction and whether H4 K20R substitutions cause defective H2AX phosphorylation and recruitment of DNA damage repair proteins.

METHODS

Cell cycle blocking experiments

Yeast grown in YPD were treated with cell cycle-blocking reagents after which they were washed in PBS and frozen dry. Yeast treated with 15µg/mL Nocodazole (Sigma M1404) were harvested after 75 minutes; G2/M-characteristic yeast budding was checked by light microscopy. Yeast treated with 10µg/mL Alpha Factor Mating Pheromone (Sigma T-6901) were harvested after at least 2 hours; Shmoo morphology was checked by light microscopy. Yeast treated with 200mM Hydroxyurea were harvested after 1 hour, 2 hours, and 3 hours. Western blots for Cdc28 (Santa Cruz sc-6709; does not vary during the cell cycle) and Clb2 (Santa Cruz sc-9071; highest at G2/M and lowest at G1) confirmed cell cycle blocks. Westerns to check H4 K20me1 levels were run such that similar total H4 amounts were loaded into each lane. Other westerns were run normalized to total WCEs.

Figure 1. H4 K20me1 levels do not vary during the cell cycle. Yeast were treated with 10µg/mL Alpha Factor Mating Pheromone (blocks in G1), 15µg/mL Nocodazole (blocks in G2/M), 200mM Hydroxyurea (blocks in S phase), or not treated (mixed population). Whole-cell extracts (WCEs) were analyzed by Western blot and probed for total H4, H4 K20me1, B-Actin, Cdc28 (does not vary during cell cycle), and Clb2 (highest during G2/M and lowest during G1). WT versus H4 K20R yeast are an antibody control. Total H4 and H4 K20me1 are probed on blots containing 1x or 2x amounts of WCEs. Samples and antibodies are respectively indicated above and to the left of each blot.

Figure 2. Assays for yeast growth during stress conditions. Dilutions of wild-type or mutant yeast were spotted onto plates conferring various stresses. (A) H4 K20R and H4 K20A substitutions did not affect growth during various stress conditions. (B) H3 K79R and H4 K20R did not demonstrate synthetic effects with DNA damage stress. (C) H4 K20R moderately rescued H3 K56R-mediated Camptothecin-sensitivity.

Figure 1

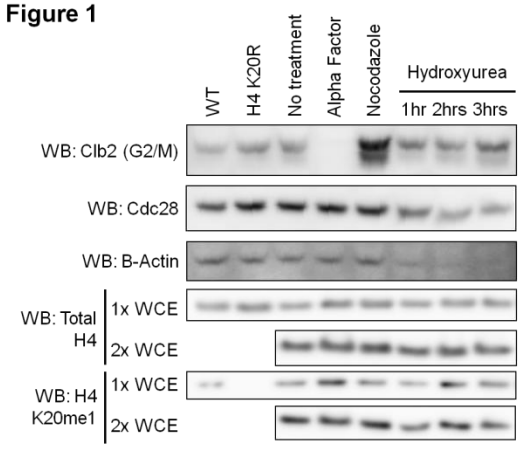
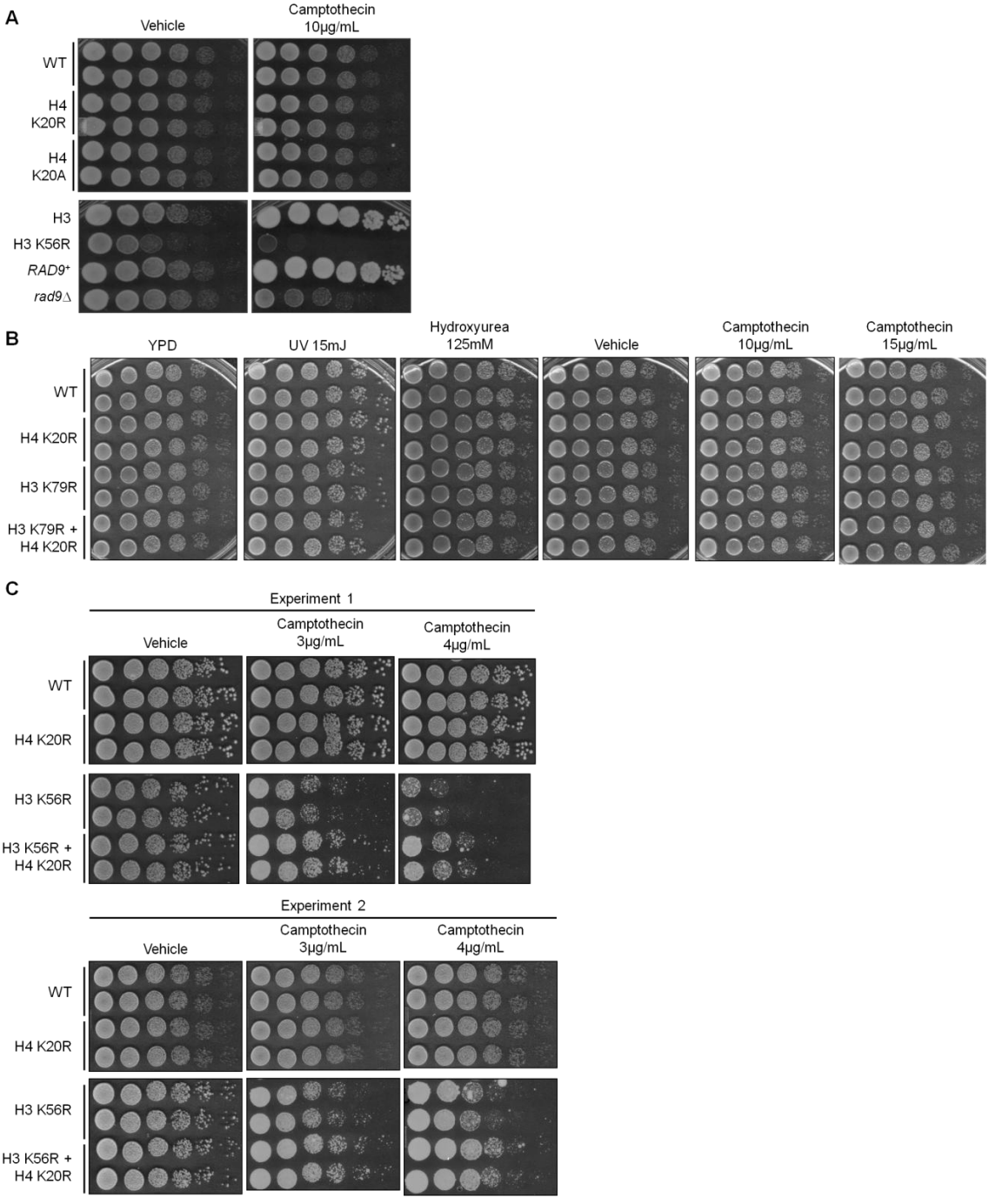

WT
H4 K20R
No treatment
Alpha Factor
Nocodazole
Hydroxyurea
1hr 2hrs 3hrs
WB: Clb2 (G2/M)
WB: Cdc28
WB: B-Actin
WB: Total H4
1x WCE
2x WCE
WB: H4 K20me1
1x WCE
2x WCE

Figure 2


A
Vehicle
Camptothecin 10µg/mL
WT
H4 K20R
H4 K20A
H3
H3 K56R
RAD9+
rad9Δ
B
YPD
UV 15mJ
Hydroxyurea 125mM
Vehicle
Camptothecin 10µg/mL
Camptothecin 15µg/mL
WT
H4 K20R
H3 K79R
H3 K79R + H4 K20R
C
Experiment 1
Vehicle
Camptothecin 3µg/mL
Camptothecin 4µg/mL
WT
H4 K20R
H3 K56R
H3 K56R + H4 K20R
Experiment 2
Vehicle
Camptothecin 3µg/mL
Camptothecin 4µg/mL
WT
H4 K20R
H3 K56R
H3 K56R + H4 K20R

DISSERTATION CHAPTER 4

NuA4 Acetylation of Spt16 in *Saccharomyces cerevisiae*

ABSTRACT

Lysine acetylation occurs on histones and participates in most chromatin-related processes. It is increasingly clear however that this modification occurs on non-histone proteins, influences their functions, and is sometimes regulated by the same acetyltransferases and deacetylases that control histone acetylation. To gain insight into the repertoire and role of *S. cerevisiae* non-histone chromatin lysine acetylations, we investigated Spt16 acetylation by the NuA4 acetyltranferase complex. Spt16 heterodimerizes with Pob3 to function as the yFACT complex. yFACT is essential and interacts with histones in multiple processes, the most well-studied of which is modulating nucleosome structure during transcription elongation. We find that NuA4 acetylates Spt16 *in vitro* and mass spectrometry determined that Spt16 overexpressed in *S. cerevisiae* was acetylated at lysines 483, 583, and 607. Loss of these acetylations by K→R substitutions was not lethal and did not demonstrate defects in intragenic transcription initiation suppression or growth defects in stress conditions. Importantly, K→R substitutions caused moderate heat-sensitivity at 39 ^{o}C whereas K→Q substitutions did not, arguing that this phenotype results from loss of acetylations rather than loss of the lysines or other modifications. The mechanism of Spt16 acetylation-mediated heat-tolerance is under investigation.

INTRODUCTION

Lysine can be acetylated on the epsilon nitrogen of its side chains. This process is proposed to involve deprotonation of the amine group's hydrogen, resulting in the nitrogen attacking the carbonyl carbon of an acetyl coenzyme-A molecule. The result is an acetyl group covalently bound to the epsilon nitrogen by its carbonyl carbon, increasing the lysine side chain's size, adding a polar carbonyl group, and reducing its net charge from positive to neutral. This modification thus extensively alters the residue's chemistry and in turn, its ability to bind other proteins, its interactions with DNA (if in a nucleosome), and the structure of the protein of which it is a part (Berndsen, C.E., 2008).

Lysine acetylation is most well-studied on histones in chromatin. This modification occurs on numerous residues of all four core histones within chromatin and on several residues on free histones. (Millar, C.B., 2006) This is connected to histone deposition, DNA-binding, reader recruitment, and chromatin structure (Shogren-Knaak, M., 2006). Depending on when it is created, where in the genome it is enriched, and on which residue and histone it is located, this modification can impact virtually every chromatin-related process.

Considering its ability to diversify the structure and modulate the function of histones, it is no surprise that lysine acetylation is increasingly indentified on non-histone proteins. For example, the mammalian chaperone Hsp90 works to adjust the folding of client proteins as a dynamic complex with other heat-shock proteins and its acetylation influences this. (Yu, X., 2002; Kovacs, J.J., 2005) The tumor suppressor p53 is acetylated at multiple residues and this affects its transcription upregulation activity (Bode, A.M., 2004). Numerous metabolic enzymes in the human liver are acetylated

(Zhao, S., 2010). Acetylation also occurs on Ku70, alpha-tubulin, Cortactin, CPS1, beta-catenin, GDH, LXR, Foxo1, and eNOS, impacting many biological processes. (Close, P., 2010; Yang, X.J., 2008) This is however a small fraction of the proteome and many more acetylations likely exist. A better understanding of them is necessary to fully understand the regulatory mechanisms that govern the cell's many pathways. Importantly, knowledge of the acetylome may allow biological or pharmacological modulators of acetylation to be used for combating diseases involving these pathways (Wanczyk, M., et. al. 2011).

The transfer of an acetyl group to histone lysines is catalyzed by histone acetyltransferases (HATs). These enzymes operate as components of multi-subunit complexes whose subunits can influence their enzymatic activity, promote enrichment within parts of the cell and along regions of the genome, allow recruitment to target proteins, allow binding to nucleosomes (in the case of histone acetylation), and confer residue specificity. These HATs and the complexes in which they reside are often conserved from yeast to humans (Lee, K.K., 2007).

One *S. cerevisiae* HAT is Essential Sas-family Acetyltransferase 1 (Esa1). One of three MYST-family acetyltransferases, Esa1 resides within the Nucleosome Acetyltransferase of Histone H4 (NuA4) complex along with twelve other subunits (Lee, K.K., 2007; Doyon, Y., 2004). NuA4 acetylates histones H4 (K5, 8, 12, and 16), H2A, and Htz1 within nucleosomes (Allard, S., 1999; Clarke, A.S., 1999; Keogh, M.C., 2006). Esa1 also exists within the less studied Piccolo NuA4 complex, consisting of Esa1 and two of the twelve NuA4 subunits (Lee, K.K., 2007). These two Esa1 complexes are thought to differently target Esa1 along the genome, with NuA4 enriched at specific locations such as active genes where it creates peaks of acetylation, and with Piccolo

NuA4 spread out along the genome, creating untargeted basal acetylation (Friis, R.M., 2009).

Esa1 is linked to many important processes. NuA4 and its H4 acetylation are associated with gene expression and thought to be enriched at promoter regions of transcribed genes (Doyon, Y., 2004), although it is recently been proposed that NuA4 also localizes to and acetylates H4 inside ORFs (Ginsburg, D.S., 2009). NuA4's HAT activity promotes *in vitro* transcription of nucleosomal DNA (Allard, S., 1999) and acetylates Htz1 K14 to promote proper kinetochore function and mitotic chromosome segregation (Keogh, M.C., 2006). NuA4 is linked to DNA damage repair; NuA4 mutants are hypersensitive to DNA damage and NuA4 acetylation is needed for DNA damage repair (Doyon, Y., 2004). Importantly, Esa1 is essential since its loss halts the cell cycle within a few cell divisions (Clarke, A.S., 1999). Although this is its most prominent phenotype, it is unknown what Esa1 must acetylate to prevent this.

While Esa1 has been studied in a mostly histone-oriented manner, it is becoming clear that its acetylation is not limited to nucleosomes. An example of this was NuA4's acetylation of one of its own components, Yng2 (Lin, Y.Y., 2008). The possible extent of its substrate repertoire was recently revealed using a proteome microarray in which *S. cerevisiae*-purified NuA4 targeted 91 out of 5,800 budding yeast recombinant proteins *in vitro* (Lin, Y.Y., 2009). One target, the gluconeogenesis enzyme Pck1, is acetylated by NuA4 *in vivo* to regulate its enzymatic activity and in-turn, yeast chronological lifespan.

Another important target revealed by this screen is the histone-interacting protein Suppressor of Ty 16 (Spt16). The gene encoding it was simultaneously and independently identified as *CDC68* (Cell Division Cycle 68), since mutant alleles confer cell cycle defects, and *SPT16*, since mutant alleles suppress *LYS2* and *HIS4*

transcription inhibition caused by insertion of a Ty transposable element within the promoter and 5' region (Rowley, A., 1991; Malone, E.A., 1991). This suppression occurs due to transcription initiation being relocated from the region disrupted by the Ty element to a downstream location within the ORF, resulting in a 5'-truncated, but functional, protein product (Malone, E.A., 1991).

Spt16 exists as a heterodimer with Pol1-Binder 3 (Pob3) and together they constitute the yeast Facilitates Chromatin Transcription (yFACT) complex or Cdc68-Pob3 (CP) complex (Wittmeyer, J., 1997; Wittmeyer, J., 1999). The DNA-binding, HMG domain-containing protein Non-Histone Protein 6 (Nhp6) weakly interacts with Spt16 and Pob3 to recruit them to chromatin and together, this heterotrimer is the Spt16-Pob3-Nhp6 (SPN) complex (Formosa, T., 2001; Stillman, D.J., 2010). Like Esa1, yFACT is essential (both components) and hypomorphic mutations cause slow-growth, a decreased maximum permissive temperature (heat-sensitivity), and transcription and DNA replication defects (Formosa, T., 2001), in addition to the suppressor of Ty (Spt) phenotype described above. It is unknown however which Spt16 function is the essential function that, when disrupted, results in inviability.

Studies of yFACT and its mammalian counterpart argue for a role in modulating nucleosomes during transcription elongation. Nucleosomes are a physical obstacle to translocating RNA polymerase 2; *in vitro* transcription by RNA polymerase 2 occurs on naked DNA but not reconstituted chromatin (Pavri, R., 2006). However, they simultaneously limit transcription initiation to places lacking nucleosomes. Inappropriately open chromatin inside some yeast genes allows RNApol2 and transcription factor recruitment to intragenic sequences that are favorable for transcription initiation but normally obscured by histones, resulting in unintended

transcripts (cryptic intragenic transcription initiation) (Carrozza, M.J., 2005; Keogh, M.C. 2005; Joshi, A.A., 2005; Li, B., 2007). Budding yeast and mammalian FACT resolve these two issues by modulating nucleosome structure during transcription elongation (independent of ATP, unlike remodellors), "loosening" their structure in-front of RNApol2 to allow translocation, and "tightening" their structure behind RNApol2 to maintain appropriate levels of DNA accessibility (Belotserkovskaya, R., 2003; Fleming, A.B., 2008; Xin, H., 2009). While this was thought to occur via the dissociation and reassociation of an H2A-H2B dimer (Belotserkovskaya, R., 2003), recent work in *S. cerevisiae* argues for a general structural reorganization of the nucleosome (Xin, H., 2009).

Although this is FACT's most well-known function, Spt16 is linked to other processes as well. Early studies of yFACT identified association with a DNA polymerase (Wittmeyer, J., 1997; Wittmeyer, J., 1999) and recent work argued that ubiquitylation of Spt16 directs it towards interacting with replication factors (Han, J., 2010). Since replication of chromatin DNA requires polymerase passage through nucleosomes, it would not be surprising if Spt16 participates here similarly to as in transcription elongation. Hypomorphic Spt16 mutants are heat-sensitive (Formosa, T., 2001), likely due to altered structure or protein interactions such that an essential function is compromised. Alternatively, Spt16 might function in induction of the heat-tolerance genes. Finally, phosphorylation of H2AX and ADP-ribosylation of Spt16 in mammals may respectively promote and inhibit H2AX-H2A exchange during DNA damage (Heo, K., 2008).

Since Spt16 is essential and linked to important processes, its acetylation by NuA4 is interesting for several reasons. First, its many functions imply that PTMs of

Spt16 might be needed to coordinate its different roles and protein-interactions. This is not surprising since ubiquitylation and ADP-ribosylation are proposed to respectively affect its roles in DNA replication and DNA damage repair (Han, J., 2010; Heo, K., 2008). Second, since NuA4 is linked to transcription, acetylation of Spt16 would imply a mechanism for this function. Third, since *ESA1*'s essential function is unknown, and since *SPT16* is also essential, it may be that Esa1's essential role in the cell is mediated through Spt16. With these implications and the *in vitro* Spt16 acetylation by NuA4 in mind, we sought to examine this acetylation *in vivo* and determine what role, if any, it has.

MATERIALS & METHODS

Generation of mutant spt16 plasmids and yeast strains

Plasmids containing wild-type or mutant *SPT16* were created as follows. The Expand High Fidelity PCR System (Roche) amplified a sequence from yeast genomic DNA spanning 1kb upstream and 500bp downstream of *SPT16+* that was then ligated into pRS313 (CEN, ARS, *TRP1*) or pRS316 (CEN, ARS, *URA3*). QuikChange site-directed mutagenesis (Agilent) sequentially created lysine substitutions (K493R K583R K607R K608R or K493Q K583Q K607Q K608Q) in the pRS313-*SPT16* vector after which sequencing confirmed fidelity.

SPT16+, *spt16 K→R*, and *spt16 K→Q* strains were created using the Delitto Perfetto two-step gene replacement system (Storici, F., 2006). Briefly, yeast with a C-terminal 3xFLAG:*KanMX* cassette integrated at endogenous *SPT16* were made diploid using a plasmid expressing the HO endonuclease after which an *HPH-GAL1prom-p53 V122A* Delitto Perfetto cassette was inserted within one of the *SPT16* ORFs, inactivating, it, but not knocking it out. pRS316 (CEN, ARS, *URA3*) containing *SPT16+* with flanking regions described above was transformed into these yeast and spores were isolated that contained plasmid-based *SPT16+* and genomic *SPT16*-3xFLAG:*KanMX* that was inactivated by the inserted Delitto Perfetto cassette. PCR products spanning the *SPT16* region containing the insert and having wild-type, K→R, or K→Q sequences were amplified from the pRS313 plasmids and transformed into these yeast. Yeast were grown on YP+Galactose plates to induce the p53 V122A counterselectable marker (selects for yeast that lost the Delitto Perfetto cassette that was inserted into the genomic *SPT16*), then SC+5FOA plates (selects for yeast that lost the *SPT16+*-bearing plasmid). Yeast were further grown on YPD plates containing

Hygromycin (indicates whether the *HPH* marker within the Delitto Perfetto cassette is present), SC-URA plates (indicates whether the *SPT16*+-bearing plasmid is present), and YPD plates containing G418 (indicates whether the C-terminal 3xFLAG:*KanMX* cassette that was at the genomic *SPT16* is present) and sequencing confirmed that the genomic *SPT16* sequence was wild-type, K→R, or K→Q, and contained the C-terminal 3xFLAG tag. The resulting strains contained the endogenous *SPT16* with a C-terminal 3xFLAG:*KanMX* cassette and either wild-type, K493R K583R K607R K608R, or K493Q K583Q K607Q K608Q sequences. Yeast with *SPT16*+, *spt16 K→R*, or *spt16 K→Q* with *his4-912δ* and *lys2-128δ* were created similarly as above but starting with endogenous *SPT16*+ without a C-terminal tag.

Identification of acetyllysines by mass spectrometry

Spt16 was purified from yeast and acetyllysines were identified by mass spectrometry by Heng Zhu's laboratory as previously described (Lin, Y.Y., 2009).

In vitro acetyltransferase reactions

In vitro acetyltransferase reactions were performed using yeast-purified NuA4, bacterially-purified substrates, and C^{14} acetyl coenzyme-A. Reactions were fractionaited on a protein gel and radiation was detected by X-ray film. All work was performed by Wendy Walter.

Yeast phenotype analyses

Assays were performed as in chapter three. Sensitivity to 39 ^{o}C was performed by either putting plates into 30 ^{o}C incubators and then slowly raising the temperature to 39 ^{o}C or

putting plates into 39 ^{o}C incubators with the plates arranged in stacks with unused plates on the top and bottom of the stacks. The was to make the temperature increase gradual rather than quick. Yeast's response to increased temperature depends on many factors including the magnitude of the change and whether the yeast were switched directly to the higher temperature or given time to adapt to a medium temperature first (Yamamoto, N., 2008). For these reasons, we found it necessary to expose the yeast to 39 ^{o}C gradually as described above rather than sharply and doing otherwise often resulted in all the yeast growing too poorly to see convincing growth phenotypes.

Detection of cryptic intragenic transcription initiation

SPT16+, *spt16 K→R*, and *spt16-197* yeast were grown at 30 ^{o}C or one hour at 39 ^{o}C after which total RNA was harvested and converted to cDNAs as described in chapter three. Random hexamers were from the Taqman Reverse Transcriptase kit. cDNAs were analyzed as described in chapter three. mRNA levels were checked with primers specific for the 3' or 5' end and 3':5' ratios were determined. Increased cryptic intragenic transcription initiation in one sample versus another would produce more mRNAs along the 3' than 5' end of the gene resulting in a larger 3':5' ratio.

RESULTS

In vitro & In vivo Evidence for Spt16 Acetylation

The proteome microarray is constructed with yeast-derived proteins (acetyltransferase activity may have co-purified with them) and the acetyltransferase reactions in the screen thus occur on an array (the surface may affect the enzyme's or substrates' conformation and reactivity) (Lin, Y.Y., 2009). Post-doctoral researchers in Shelley Berger's laboratory therefore followed-up traditional (in solution) *in vitro* acetylation reactions with C^{14} acetyl-coenzyme A, yeast-purified NuA4, and bacterially-purified recombinant proteins identified from the proteome microarray. Reactions were fractionaited with a protein gel after which X-ray film detected radioactivity (Figure 1A). All reactions containing substrates, including r. Spt16, had radioactive bands that were absent in NuA4-only reactions and corresponded to their substrates' expected sizes.

To determine which Spt16 lysines are acetylated, researchers in Heng Zhu's lab purified overexpressed Spt16 from *S. cerevisiae* and used mass spectrometry to identify acetyllysines as previously described (Lin, Y.Y., 2009). Lysines 493, 583, and 607 were identified as being acetylated (Figure 1B). Since these three sites are clustered around the middle of Spt16, we wondered what significance this regions holds. Despite this protein's importance, there is limited understanding of what regions contribute to what function. Hypomorphic mutations created by random mutagenesis are not confined to distinct regions of Spt16 and these mutations' phenotypes generally do not correlate with their locations (Formosa, T., 2001). Spt16 can be organized into N-terminal, middle, and C-terminal folding domains, a region within the middle of the protein is necessary and sufficient for heterodimerization with Pob3 (O'Donnell, A.F., 2004; VanDenmark, A.P., 2006), and the N-terminal region contains a histone tail-binding, catalytically inactive,

aminopeptidase domain that is dispensable for nucleosome binding and cell viability (VanDenmark, A.P., 2008). Interestingly, all three acetyllysines reside within or adjacent to the Pob3-heterodimerization domain (Figure 1B) and thus might affect Pob3-interactions and in turn, Spt16's functions.

Functional Analyses of spt16 K→R Substitution Strains

To determine if these acetyllysines influence Spt16's functions, we generated yeast strains in which the genomic *SPT16* was mutated to convert the three lysines to unacetylatable arginines. Since acetyllysines can sometimes exert their effects in a synergistic fashion (Yang, X.J., 2008), we chose to examine simultaneous substitutions of all three lysines (*spt16 K493R K583R K607R K608R*; *spt16 K→R*). K608 was mutated in-case it gets targeted in the absence of K607. The *spt16 K→R* mutant strain was viable and grew similarly to the *SPT16*+ strain at 30 ^{o}C (Figure 2A, B) and similar results were seen with a *SPT16* plasmid shuffle strain (data not shown). To test whether acetylation participates in Spt16's known functions, we compared growth of *SPT16*+ and *spt16 K→R* strains under stress conditions. We checked growth with galactose as the carbon source or without arginine since Spt16 participates in transcription of *GAL1* (Fleming, A.B., 2008) and arginine synthesis genes (Ginsburg, D.S., 2009), growth with Hydroxyurea since Spt16 is linked to DNA replication (Wittmeyer, J., 1997; Wittmeyer, J., 1999; Han, J., 2010), and growth with UV light. Wild-type and mutant yeast grew similarly in all these conditions (Figure 2A, B).

Since yFACT participates in transcription elongation, in-part to maintain sufficient intragenic compaction (Fleming, A.B., 2008), we checked whether *spt16 K→R* yeast haveincreased intragenic cryptic transcription initiation. The temperature-sensitive yeast

strain *spt16-197* (G132D) had increased intragenic transcription initiation at four genes at 39 ^{o}C, indicated by increased 3' versus 5' qPCR signals at those mRNAs (Figure 3). The *spt16 K→R* allele however did not produce detectable intragenic transcripts at these genes. Since Spt16 mutations confer the spt phenotype (suppression of *LYS2* and *HIS4* transcription defects resulting from Ty element insertions at their promoters) (Malone, E.A., 1991), we asked whether the *spt16 K→R* allele confers such an effect. The spt phenotype can be assessed using yeast where a Ty element insertion in the *LYS2* and *HIS4* 5' regions inhibits their transcription, causing dependence on lysine and histidine-supplemented media. While the *spt16-197* allele in this strain rescued lysine and histidine auxotrophy, the *spt16 K→R* allele caused no such effect (data not shown).

Analyses of spt16 K→R Substitution Strain Heat-Sensitivity

Since many *SPT16* mutations are sensitive to elevated temperatures (Formosa, T., 2001), we checked whether loss of these acetylatable lysines conferred heat sensitivity. Compared to *SPT16*+ yeast, *spt16 K→R* yeast grew similarly well at 37 ^{o}C (Figure 2A) but less well at 39 ^{o}C (Figure 2B, C). Importantly, yeast with these lysines changed to the acetyllysine-mimic glutamine (*spt16 K493Q K583Q K607Q K608Q*; *spt16 K→Q*) did not demonstrate a growth defect relative to *SPT16*+ yeast at 39 ^{o}C (Figure 2B, C), arguing that the *spt16 K→R* heat-sensitivity resulted from loss of acetylation rather than loss of the lysines or unidentified non-acetyl modifications at these sites.

There are several ways that Spt16 acetylation loss at these sites could cause temperature-sensitivity. Since yFACT participates in transcription, loss of Spt16 acetylation could compromise the induction of heat-shock response genes, leading to defective heat-tolerance. Alternatively, since yFACT is thought to participate in

transcription initiation and elongation of many genes and DNA replication, specific interactions involving Spt16 that are necessary for these processes but sensitive to high temperatures might be compromised at 39 ^{o}C if not for stabilizing effects by the acetylation. Similarly, Spt16's general structure might be compromised at 39 ^{o}C, leading to general loss-of-function, if not for stabilization by the acetylation. Considering that Spt16 functions as a heterodimer with Pob3 and that all three acetyllysines reside at or adjacent to the Pob3-interaction region, it is possible that this critical interaction is dependent on these acetylations to maintain integrity at 39 ^{o}C.

We first asked whether the *spt16 K→R* allele confers defective upregulation of heat-response genes during growth at 39 ^{o}C. Yeast respond to elevated temperatures by several mechanism, one of which is the induction of genes whose products help the cell resist the detrimental effects of excess heat (Yamamoto, N., 2008). mRNA levels were checked from *SPT16+* or *spt16 K→R* yeast that were grown at 30 ^{o}C or for 15, 30, or 60 minutes at 39 ^{o}C. In wild-type yeast, *SSA3*, *BTN2*, *FES1*, and *HSP78*, Heat-Shock Factor 1-targets that are upregulated in response to high temperatures, were induced by 15 minutes at 39 ^{o}C, whereas *ACT1* and *SWI6*, genes not related to the heat-shock response, showed no major changes in their mRNA levels (Figure 4). No differences were observed however between these yeast and *spt16 K→R* yeast in the levels of these mRNAs.

To check whether the K→R substitutions cause defects in particular Spt16 functions at elevated temperatures, we checked whether this mutant strain had increased cryptic transcription initiation at elevated temperatures. While the *spt16-197* strain had increased cryptic transcripts at 39 ^{o}C compared to at 30 ^{o}C or to wild-type

strains at 39 °C, the *spt16 K→R* strains had unchanged levels of intragenic transcription initiation at four genes checked at 39 °C (Figure 3).

DISCUSSION

Spt16 Functional Regulation by Acetylation

To determine if loss of these acetylations compromises particular Spt16 functions, we will ask if additional Spt16-regulated processes are defective with the K→R substitutions at 39 °C compared to wild-type strains. Although we have checked for spt16 K→R's functionality at 39 °C in intragenic transcription initiation suppression, we have only looked at a small number of genes. Numerous other cryptic transcripts exist (Li, B., 2007) and an unbiased examination of intragenic transcription initiation would better reveal the K→R substitutions' impact on yFACT's regulation of intragenic DNA accessibility genome-wide. Were Spt16 acetylation needed to maintain yFACT's cryptic transcription initiation suppression at high temperatures, the numerous inappropriate transcriptions initiated at 39 °C due to the lack of acetylation might titrate out the transcription machinery, or create a bottleneck in mRNA processing pathways, compromising cell viability.

Another possibility is that the acetylation is needed at 39 °C for yFACT's nucleosome modulation activity that facilitates RNA polymerase passage. To examine this, we will use ChIP to ask whether the decrease in intragenic histones that occurs upon gene induction (Fleming, A.B., 2008) is compromised in K→R versus wild-type *SPT16* yeast at 39 °C. As an alternative to checking histone levels by ChIP, we could use MNase assays to observe changes in the chromatin compaction. Since yFACT is linked to DNA replication and may have other roles, it is possible that the acetylation is

needed for these functions at 39 ^{o}C. We also note that the decreased viability at 39 ^{o}C with the K→R substitutions may result from the combination of many impaired Spt16 functions. For example, defects in intragenic transcription suppression combined with defects in RNA polymerase passage, DNA replication, and other Spt16 functions, might lower viability.

Since all three acetyllysines reside in or around Spt16's Pob3-heterodimerization region (Figure 1B), these marks may influence this interaction, although whether these residues lie at the Spt16-Pob3 interface is unclear due to a lack of yFACT structural data. They might for example stabilize the Pob3 interaction in a way that is most important during elevated temperatures. Were this true, yFACT would have decreased integrity at 39 ^{o}C with the K→R substitutions relative to wild-type Spt16, resulting in all its functions being compromised. Another possibility is that the acetylations directly affect Spt16's structural stability, counteracting the destabilizing effects of heat.

Observing Acetylation of Endogenous Spt16

A critical remaining experiment is to observe these acetylations *in vivo* with endogenous Spt16 levels (the mass spectrometry identified *in vivo* acetylation with overexpressed *SPT16*). Three methods exist for identifying acetylations *in vivo*: mass spectrometry, pan-acetyl antibodies, and site-specific acetyl antibodies. Mass spectrometry can identify unknown sites but may require large amounts of protein. Pan-acetyl antibodies can also detect unknown sites, but identifying the residues requires making potentially many lysine substitution mutations and the binding can never be completely sequence-independent meaning some acetylations might be missed. Site-specific antibodies have sequence-specificity but take the longest time to acquire and must be generated for

each acetylation. Our studies identified the sites via mass spectrometry analysis of overexpressed yeast-purified proteins. We then attempted to confirm these acetylations *in vivo* on endogenous Spt16 using pan-acetyl antibodies and the K→R substitutions. We purified total Spt16 by FLAG-affinity purification (genomic location with C-terminal FLAG tag) and then did westerns with multiple pan-acetyl antibodies. Unfortunately, these methods did not confirm the marks' presence *in vivo* (data not shown). As described above, site-specific acetyl antibodies may be necessary to confirm these marks using endogenously expressed *SPT16*. Alternatively, since custom antibodies do not always work, and since three such antibodies would need to be generated, mass spectrometry could be utilized to confirm these marks if sufficient non-overexpressed Spt16 were purified. We believe our work argues for the acetylations' existence on Spt16 at endogenous levels since the heat sensitivity with the K→R substitutions was not seen with the K→Q substitutions.

Immediate Future Directions

Future studies of Spt16 acetylation will focus on confirmation of *in vivo* acetylation of endogenous levels of Spt16 using mass spectrometry or site-specific anti-acetyllysine antibodies. We note that if the cell requires these acetyllysines at 39 ^{o}C, then detection may be necessary at elevated temperatures. To determine the mechanism of K→R-mediated heat-sensitivity, we will purify Spt16 and observe what *in vivo* interactions at 39 ^{o}C are altered by loss of acetylation. We will also check cryptic intragenic transcription initiation in an unbiased fashion (expression microarrays), chromatin structure at genes (MNase assays), and nucleosome modulation at genes (ChIPs for total histones) between wild-type and Spt16 K→R yeast at 39 ^{o}C to elucidate the

mechanism. We believe that these continuing studies will increase the repertoire of known non-histone chromatin acetylation targets, elucidate a role of NuA4, and reveal a mechanism of regulation of an important chromatin protein.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Spt16 acetylation

(A) Acetylation reactions were performed with yeast-purified NuA4, bacterially-purified recombinant substrates, and radioactive acetyl-coenzyme A. Reactions were fractionaited on a polyacrylamide gel and radioactivity was assessed by autoradiography. Protein sizes and reactions are indicated to the left of and above each gel. Red arrow indicates r. Spt16. (B) Map of Spt16 (1,035aa). Grey bar represents Spt16. White boxes represent folding and Pob3-interaction domains as reported in VanDenmark, A.P., 2008. Black boxes represent selected domains reported at yeastgenome.org. Amino acids are indicated in parentheses. Arrows indicate acetyllsyines.

Figure 2. Spt16 mutant growth assays

Serial dilutions of *SPT16+, spt16 K→R*, or *spt16 K→Q* yeast were spotted onto plates with different growth conditions. K→R indicates K493R K583R K607R K608R. K→Q indicates K493Q K583Q K607Q K608Q. (3) indicates K583, K607, and K608 mutated. H3 versus H3 K56R, *SPT16+* versus *spt16 G132D*, and *ESA1+* versus *esa1 L254P* are positive controls for stress conditions.

Figure 3. *spt16 K→R* substitutions do not cause cryptic transcription initiation

Yeast with *SPT16+* or *spt16 K493R K583R K607R K608R* were grown at 30 ^{o}C or 39 ^{o}C after which total RNA was extracted. Ratios of levels of 3' versus 5' ends of mRNAs indicate cryptic transcription initiation. *Spt16 G132D* strains have increased intragenic transcription initiation at 39 ^{o}C.

Figure 4. *spt16 K→R* substitutions do not prevent heat-shock gene induction

Yeast with *SPT16+* or *spt16 K493R K583R K607R K608R* were grown at 30 ^{o}C or 39 ^{o}C after which total RNA was extracted.

Figure 1

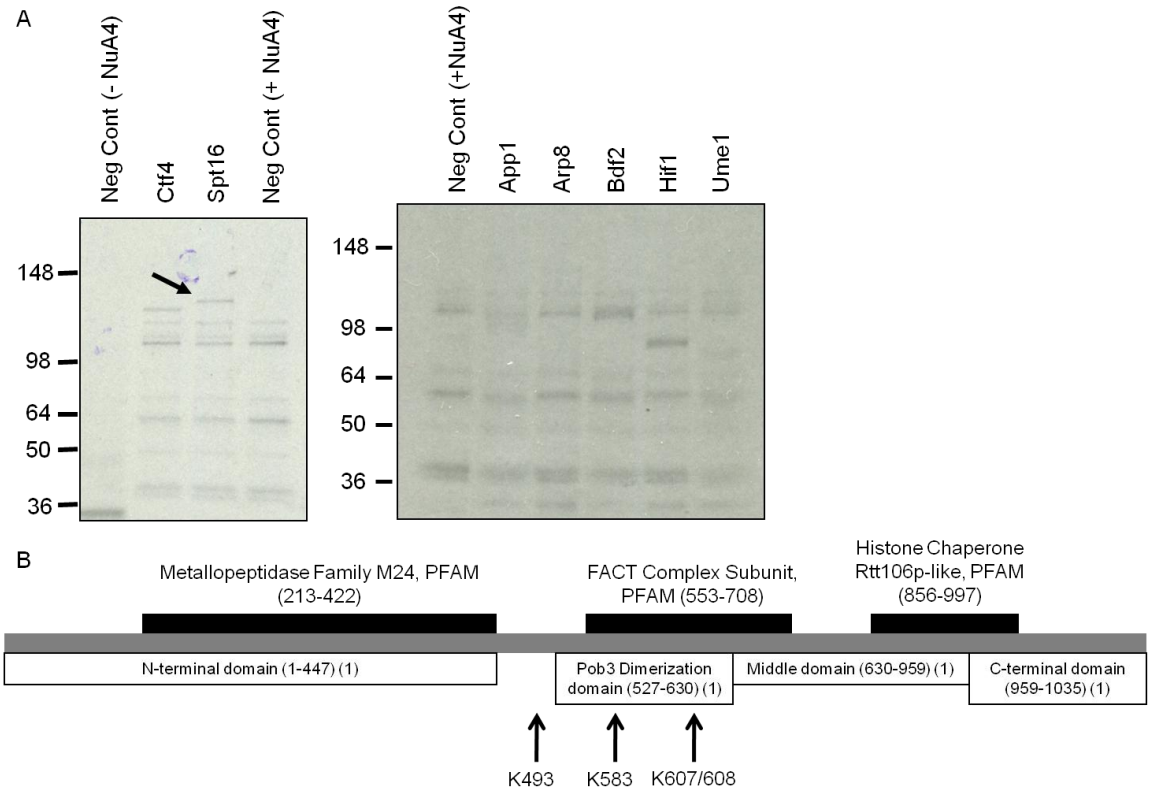
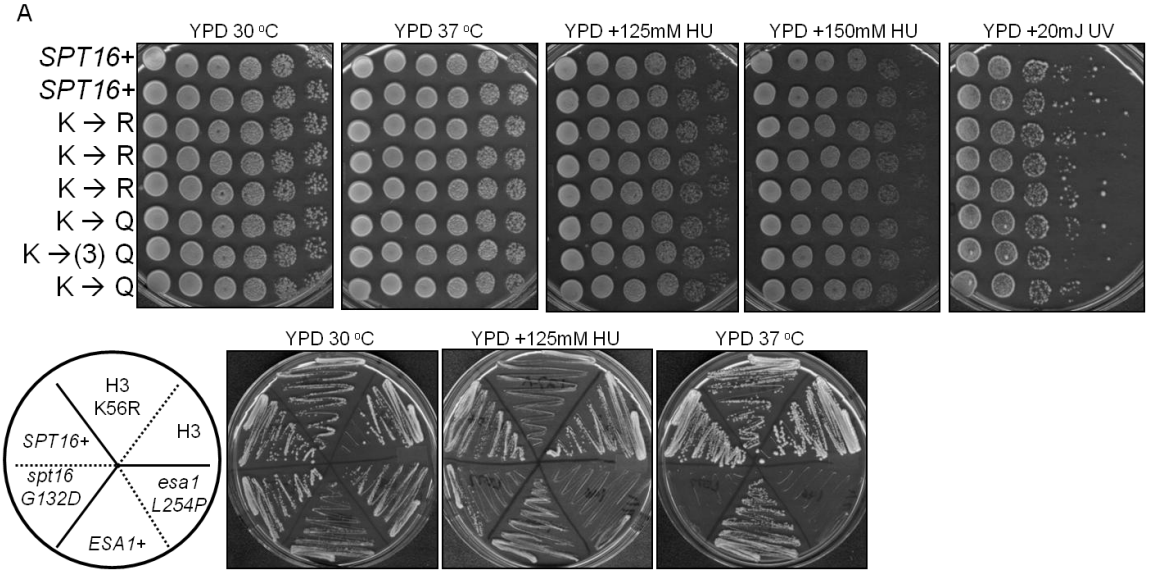


Figure 2



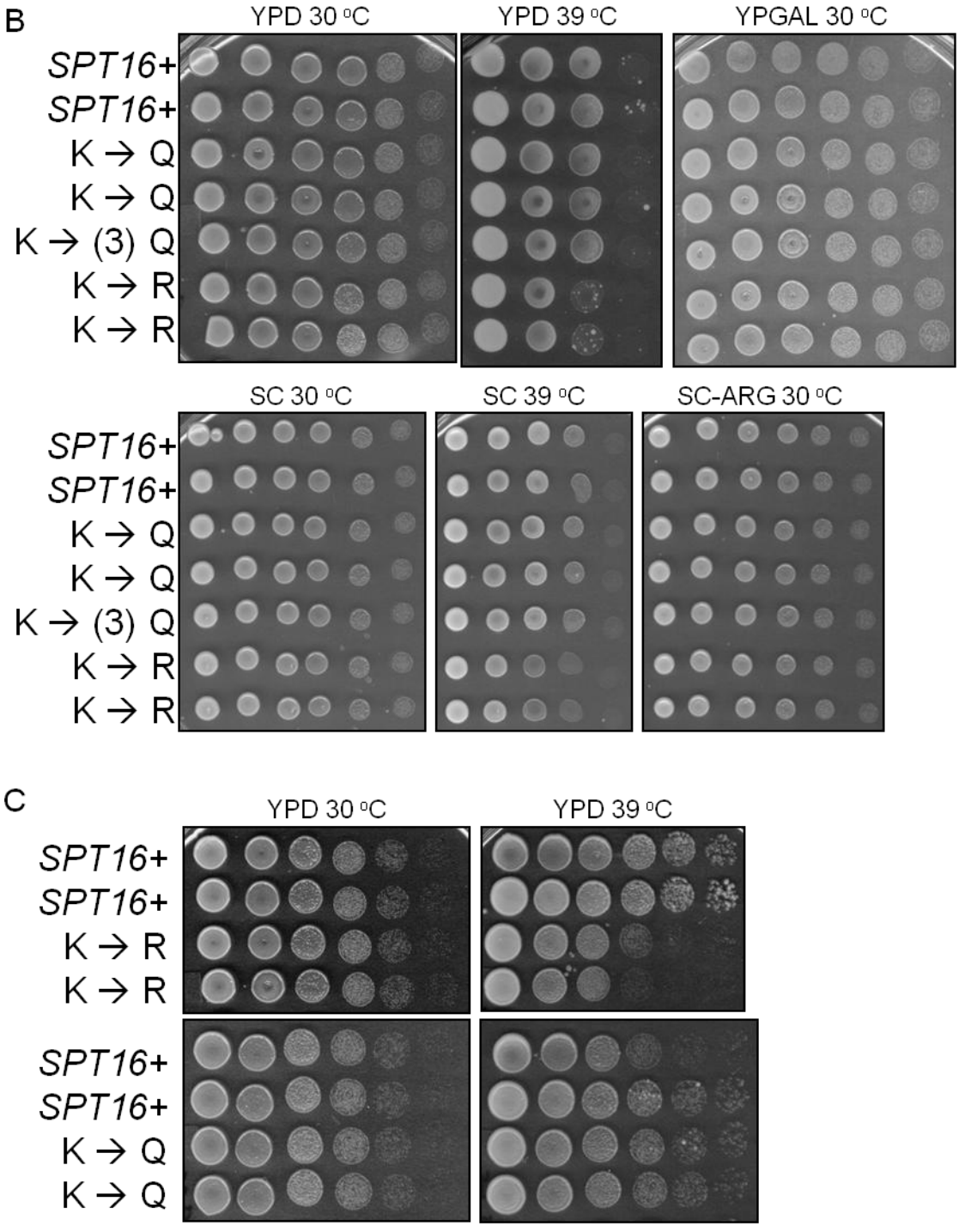

B
YPD 30 °C
YPD 39 °C
YPGAL 30 °C
SPT16+
SPT16+
K → Q
K → Q
K → (3) Q
K → R
K → R
SC 30 °C
SC 39 °C
SC-ARG 30 °C
SPT16+
SPT16+
K → Q
K → Q
K → (3) Q
K → R
K → R
C
YPD 30 °C
YPD 39 °C
SPT16+
SPT16+
K → R
K → R
SPT16+
SPT16+
K → Q
K → Q

Figure 3

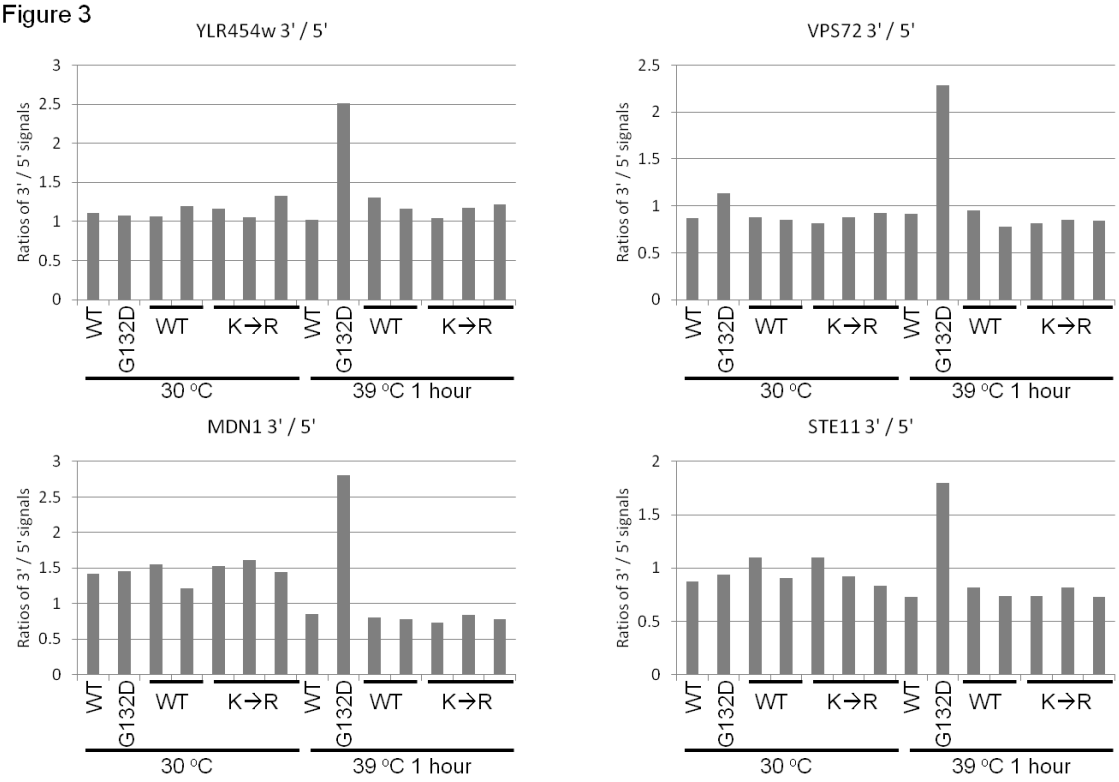

YLR454w 3' / 5'
VPS72 3' / 5'
MDN1 3' / 5'
STE11 3' / 5'
Ratios of 3' / 5' signals
WT
G132D
WT
K→R
WT
G132D
WT
K→R
30 °C
39 °C 1 hour

Figure 4

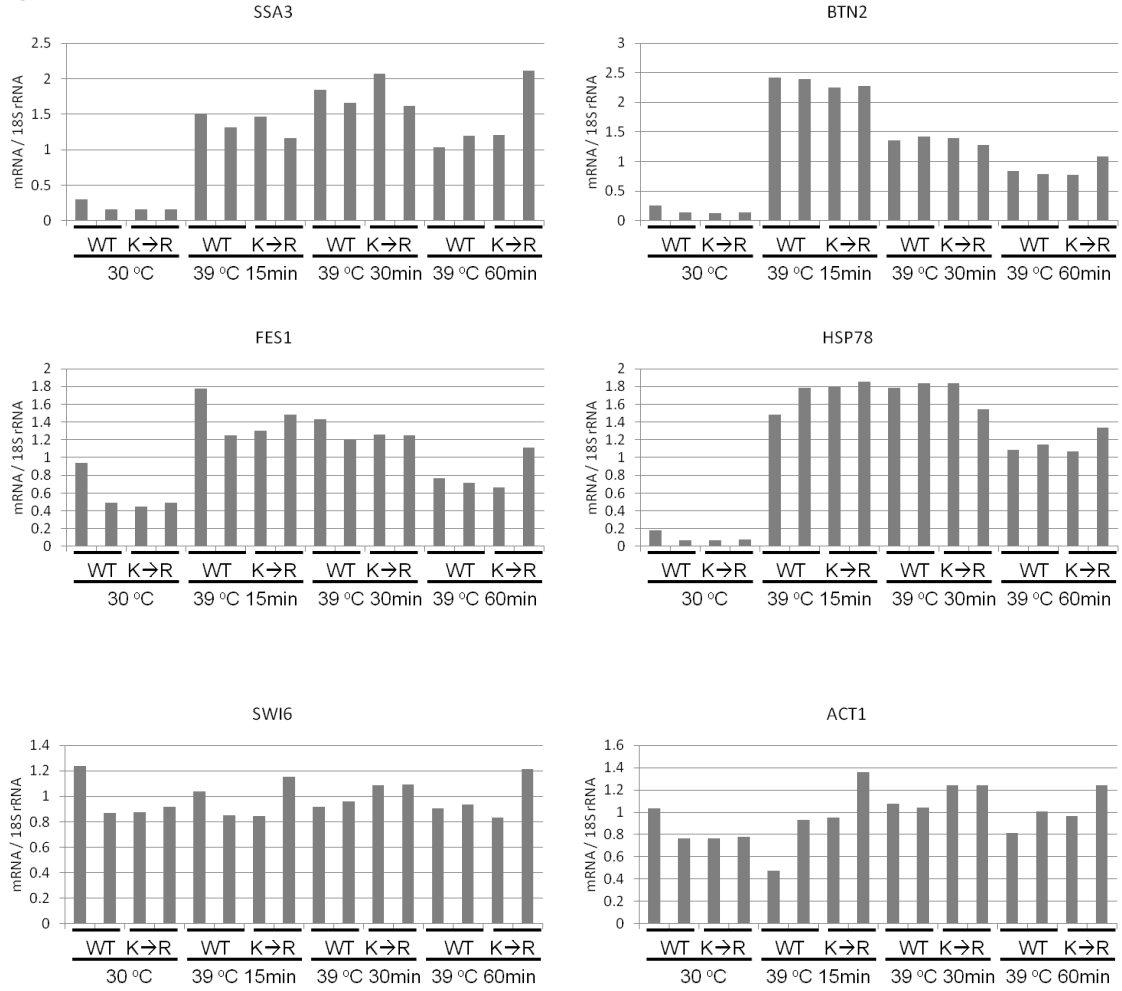

SSA3
BTN2
FES1
HSP78
SWI6
ACT1
mRNA / 18S rRNA
WT K→R
30 °C
39 °C 15min
39 °C 30min
39 °C 60min

Table 1. Yeast strains used in this study.

Figure 2A	Genotype	Source
YCE345	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 SPT16-3xFlag:KanMX	This study
YCE346	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 spt16 K493, 583, 607, 608R-3xFlag:KanMX	This study
YCE348	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 spt16 K493, 583, 607, 608Q-3xFlag:KanMX	This study
YCE349	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 spt16 K583, 607, 608Q-3xFlag:KanMX	This study
YCE074	MATa his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2−128δ (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 with pRM204 [HHT2, HHF2, CEN, TRP1]	This study
YCE127	MATa his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2−128δ (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 pCE024 [pRM204 *hht2*-K56R, HHF2 CEN, TRP1]	This study
FY56	Mat alpha his4-912δ lys2-128δ ura3-52	Malone, E.A., 1991
L577	Mat alpha his4-912δ lys2-128δ ura3-52 spt16-197	Malone, E.A., 1991
LPY3498	MATa his3Δ200 leu2-3,112 trp1Δ1 ura3-52 ESA1	Clarke, A.S., 1999
LPY3500	MATa his3Δ200 leu2-3,112 trp1Δ1 ura3-52 esa1::HIS3 esa1-L254P:URA3	Clarke, A.S., 1999
Figure 2B	Genotype	Source
YCE345	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 SPT16-3xFlag:KanMX	This study
YCE346	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 spt16 K493, 583, 607, 608R-3xFlag:KanMX	This study
YCE348	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 spt16 K493, 583, 607, 608Q-3xFlag:KanMX	This study

YCE349	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 spt16 K583, 607, 608Q-3xFlag:KanMX	This study
Figure 2C	Genotype	Source
YCE345	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 SPT16-3xFlag:KanMX	This study
YCE346	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 spt16 K493, 583, 607, 608R-3xFlag:KanMX	This study
YCE348	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 spt16 K493, 583, 607, 608Q-3xFlag:KanMX	This study
Figure 3	Genotype	Source
FY56	Mat alpha his4-912δ lys2-128δ ura3-52	Malone, E.A., 1991
L577	Mat alpha his4-912δ lys2-128δ ura3-52 spt16-197	Malone, E.A., 1991
YCE345	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 SPT16-3xFlag:KanMX	This study
YCE346	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 spt16 K493, 583, 607, 608R-3xFlag:KanMX	This study
Figure 4	Genotype	Source
YCE345	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 SPT16-3xFlag:KanMX	This study
YCE346	MATa HIS3-200 LEU2-3,112 trp1-1 URA3-52 spt16 K493, 583, 607, 608R-3xFlag:KanMX	This study

DISSERTATION CHAPTER 5

Discussion

Section 1 Summary of our goals

We chose to use *S. cerevisiae* to study chromatin PTMs since it provides technological advantages over higher eukaryotic model organisms including the critical ability to generate amino acid substitutions in histones and other proteins. We chose to focus on lysine's PTMs since they are the most varied. We also chose to focus primarily, though not solely, on lysine methylation since this is its most complex and functionally diverse modification and since its previously discovered reversibility made it an important area of investigation. Finally, we chose to investigate chromatin lysine modifications through three avenues of research: the lysine PTM enzymes, the histone chromatin lysine PTMs, and the non-histone chromatin lysine PTMs.

Our studies of Saccharomyces cerevisiae chromatin post-translational modifications

1) Histone Lysine Demethylation in *S. cerevisiae*:

Histone lysine methylation was long considered permanent due to the lack of an identified demethylase. Reversibility was demonstrated however when mammalian LSD1 and JHDM1 were shown to demethylate histone methyllysines *in vitro* and *in vivo* via an amine oxidase and JmjC domain respectively. Since JmjC domains were predicted in most model organisms and expected to be an important aspect of chromatin regulation (Klose, R.J., 2006), we chose to characterize the JmjC protein Kdm5, encoded by *S. cerevisiae JHD2*.

2) Histone Lysine Methylation in *S. cerevisiae*:

H4 K20 is mono-, di-, and trimethylated in many higher eukaryotes. The monomethylated state is especially interesting since it seems to be distinct from the other states in terms of the responsible enzymes, genomic enrichment, reader proteins, and biological functions. However, occasionally conflicting reports and the large number of functions make its overall role not fully clear (Brustel, J., 2011; Yang, H., 2009). Further, studies involving perturbing its methyltransferase Set8 (Pr-Set7) are complicated by the fact that this may alter the di- and trimethylated states and the fact that Set8 targets p53 and perhaps other proteins (Shi, X., 2007). The genetically tractable *Schizosaccharomyces pombe* also possess H4 K20me1, but using it as a model to clarify H4 K20me1's role is limited since H4 K20me2, 3 are also present, the same enzyme may create all three states, and functional differences between the states are unclear (Sanders, S.L., 2004).

Unlike most higher eukaryotes, *S. cerevisiae* was considered to lack methylation of H4 K20, as well as H3 K9 and H3 K27. These three lysines are conserved from budding yeast to humans and their methylation in higher eukaryotes is associated with transcriptional repression or chromatin compaction. In contrast, lysines H3 K4, H3 K36, and H3 K79 and their methylation are conserved from budding yeast to humans and this methylation is associated with transcriptional activity or an open chromatin state (Martin, C., 2005). *S. cerevisiae* thus possess all six conserved lysines and the three "active" methylations but lack the three "repressive" methylations. As such, budding yeast are considered to have chromatin that is generally more "open" than higher eukaryote chromatin.

Since a mass spectrometry report suggested that monomethylated H4 K20 exists in *S. cerevisiae* (Garcia, B.A., 2007), we chose to confirm and characterize this mark for several reasons. First, since budding yeast are excellent for studying histone PTMs, and since the di- and trimethylated states may be absent, unlike in *S. pombe*, this would be an opportunity to clarify the role of this particular methylation state of this lysine. Second, if this mark were associated with repression, this would be the first repressive *S. cerevisiae* histone methyllysine.

3) NuA4 Acetylation of Spt16 in *S. cerevisiae*:

It is increasingly clear that PTMs are employed by the cell to regulate proteins in-general. Since chromatin is comprised of many non-histone proteins and is replete with protein-modifying enzymes, it is expectable that modifications occur on and regulate these non-histone proteins. The scope of this for acetylation was demonstrated recently using a budding yeast proteome microarray. The HAT NuA4 *in vitro* acetylated 91 proteins, many of which were chromatin-related (Lin, Y.Y., 2009). One *in vitro* target was Spt16, part of the yFACT complex that is essential and participates in transcription elongation and possibly DNA replication and repair (Doyon, Y., 2004). Since this would be an opportunity to expand the repertoire of non-histone acetylation targets *in vivo*, shed light on NuA4's function, and elucidate the regulation of a critical chromatin protein, we chose to confirm and characterize the acetylation of *S. cerevisiae* Spt16.

Section 2 Histone Lysine Demethylation in *Saccharomyces cerevisiae*

S. cerevisiae Kdm5 demethylates mono-, di-, and trimethylated H3 K4 in vitro

The JmjC domain-containing proteins from major model organisms can be organized into groups based on phylogenetic analysis and domain architecture (Klose, R.J., 2006). Kdm5 (Yjr119c in the review) is a member of the JARID group. Noting that another JARID group member, mammalian Jarid1d, demethylates methylated H3 K4 (Lee, M.G., 2007) and is orthologous to Kdm5 especially in terms of the predicted catalytically essential residues, we hypothesized that Kdm5 also demethylates methylated H3 K4. Using *in vitro* demethylase assays with core histones and peptides and *in vivo* overexpressions of *JHD2* combined with western analyses, we demonstrated the following about Kdm5's activity. First, Kdm5 can demethylate the mono-, di-, and trimethylated states of H3 K4 *in vitro*. Second, Kdm5's activity is site-specific since we did not detect H3 K36 or H3 K79 demethylation *in vitro* and since overexpressing *JHD2* did not change H3 K36me3 levels *in vivo*. Third, Kdm5 can target peptides and core histones *in vitro*, but may not be able to target nucleosomes effectively *in vitro*. Fourth, Kdm5 demethylates H3 K4me2, 3 *in vitro* such that the mono- and dimethylated states can accumulate, presumably as intermediates.

S. cerevisiae Kdm5 negatively regulates Set1 and H3 K4 methyl levels at GAL1 during induction & repression

While I contributed the previously described *in vitro* work, another graduate student, Kristin Ingvarsdottir, characterized Kdm5 *in vivo* by using ChIP to observe H3 K4me1-3 and Set1 levels at the *GAL1* 5' and 3' regions during its induction and subsequent

repression. Set1, as part of the COMPASS complex, is the HMT responsible for H3 K4me1-3 (Shilatifard, A., 2008) and any analysis should consider that changes in H3 K4 methylation could result from changes in Set1/COMPASS recruitment. Kdm5 loss by *JHD2* deletion did not cause noticeable changes to Set1 or H3 K4me1-3 levels globally. Changes however were noticeable at *GAL1* during its induction and subsequent repression and can be summarized in the following three points.

First, during induction in wild-type yeast, Set1 levels increase at the *GAL1* 5' but not 3' region. Without Kdm5 however, Set1 is now recruited to both the 5' and 3' regions during induction and the increase at the 5' region is of greater magnitude. This suggests that Kdm5 inhibits Set1 recruitment to *GAL1* during induction, partially limiting 5' recruitment and completely preventing 3' recruitment.

Second, during induction in wild-type yeast, H3 K4me1-3 increases at the 5' but not at the 3' regions, consistent with Set1 recruitment patterns. Further, at the 5' region, while all three methyl states increase upon *GAL1* expression, the increase is largest for K4me3 and smallest for K4me1. Without Kdm5 however, *GAL1* induction is accompanied by an increase of all three methyl states at both the 5' and 3' regions. Further, at the 5' region during *GAL1* induction, the increase in K4me3 is only slightly larger than in wild-type yeast, but the increase in K4me1, 2 is much larger than in wild-type yeast. This suggests that Kdm5 opposes H3 K4 methylation at *GAL1* during induction, but just like with Set1 recruitment, it is a little different at the 5' and 3' regions. At the 5' region during induction, all three H3 K4 methyl states try to increase greatly but Kdm5 suppresses the lower methyl states moreso than the higher methyl states such that K4me1 increases the least and K4me3 increases the most. At the 3' region during induction, Kdm5 suppresses H3 K4me1-3.

Third, upon repression of active *GAL1* in wild-type yeast, H3 K4me1-3 are present at the 5' region and K4me3 in particular shows a decrease over time. Without Kdm5 however, K4me3 levels are a little higher (as mentioned above) and decrease slower.

Model of Kdm5 function at GAL1

Upon *GAL1* induction, Set1 is recruited to the gene to increase levels of H3 K4me1-3, which then function in transcription. Kdm5 simultaneously inhibits this methyllysine increase, both directly by demethylating the histones, and indirectly by limiting Set1's recruitment or activity. Since lysine methylation regulates some non-histone proteins, and since Set1 exists within and is regulated by the COMPASS complex (Shilatifard, A., 2008), Kdm5 might limit Set1's recruitment by demethylating a methyllysine on Set1 or any other COMPASS component. Likewise, upon gene repression, Kdm5 promotes a decrease in the 5' region H3 K4me3 levels, likely directly by removing the methyl groups and perhaps indirectly by promoting the dissociation of Set1/COMPASS.

An interesting question is the regulation of Kdm5's activity at *GAL1* during gene expression and repression. Kdm5 is presumably recruited to or away from *GAL1* to regulate H3 K4 methyl levels. ChIP however failed to detect Kdm5 enrichment or depletion at *GAL1* during any change in gene expression (our unpublished results). This might be an artifact of our ChIP methods or due to poor Kdm5 crosslinking ability or transient interactions. It might however be a general property of JmjC proteins since others have reported failure to observe enrichment of two budding yeast H3 K36me-specific JmjC proteins at specific genes despite those proteins affecting H3 K36me levels at those genes. Alternatively, Kdm5 may be broadly distributed along chromatin

and its functioning at specific places and times might be regulated by its own PTM or the recruit of cofactors. Another possibility, if Kdm5 regulates H3 K4me levels at *GAL1* only indirectly through demethylating Set1/COMPASS recruitment, is that Kdm5 interacts with Set1/COMPASS without binding chromatin.

Kdm5 protein interactions

Little is known about Kdm5's *in vivo* protein interactions and we and others failed to observe proteins copurifying with yeast-purified Kdm5 (Liang, G., 2007; our unpublished results). There are however several reason to suspect that many interesting Kdm5-protein interactions exist *in vivo*. First, the E3 ubiquitin ligase Not4 ubiquitylates Kdm5 *in vivo* to regulate its stability (Mersman, D.P., 2009; Huang, F., 2010). That this interaction was missed by us and others demonstrates that failure to observe proteins copurifying from yeast with Kdm5 does not mean that *in vivo* interactions do not occur. We note that these copurification experiments were performed under "normal" growth conditions and some Kdm5-protein interactions may only occur during stress conditions.

Second, Kdm5 by itself may not be capable of targeting nucleosomes *in vitro*; we failed to see such activity with insect cell-purified Kdm5 (data not shown), others only reported using histone substrates (Liang, G., 2007), and notably, the mammalian ortholog Jarid1d cannot target nucleosomes *in vitro* (Lee, M.G., 2007), arguing that this may be a general property of JARID family members. Kdm5 may thus rely on adaptor proteins to facilitate interactions with nucleosomes and as an example, mammalian LSD1 demethylates histones by itself but requires CoREST to demethylate nucleosomes (Lee, M.G., 2005). We note a recent report that Myc-tagged Kdm5 bound nucleosomes *in vitro*. These nucleosomes were yeast-purified however and additional binding factors

may have been present (Huang, F., 2010). Third, ChIP has not revealed Kdm5 enrichment at *GAL1* (our unpublished results) despite regulating its methylation and similar observations are reported for the JmjC proteins Jhd1 and Rph1 at other genes (Kim, T., 2007). It thus may be that Kdm5 interacts with adaptor proteins that facilitate its nucleosome-binding *in vivo* and prevent efficient ChIP crosslinking to histones.

It would be interesting to identify additional Kdm5-interacting proteins since this would give insights into its regulation and possibly reveal additional biological roles. Since standard Kdm5-purifications from yeast under optimal growth conditions have revealed little, purifications under various stress or nutritional conditions may be more informative. Noting that a budding yeast proteome microarray was created a few years ago and successfully used in an *in vitro* acetyltransferase screen (Lin, Y.Y., 2009), it is tempting to speculate that such technology could be used to identify proteins that Kdm5 directly binds (Not4 or H3 could be a positive control). Previous attempts to find Kdm5 interactors used epitope tags that may have interfered with interactions (Liang, G., 2007; our unpublished results). Purifications of physiological Kdm5 with anti-Kdm5 antibodies would thus be useful for future experiments.

The JmjC protein Gis1, which has no reported demethylase activity *in vitro*, has JmjC and JmjN domains capable of protein interactions according to a two-hybrid experiment (Tronnersjo, S., 2007), and the other JmjC proteins would thus be expected to interact with many proteins as well. We note however that purifications of two other *S. cerevisiae* JmjC proteins, the H3 K36 demethylases Jhd1 and Rph1, also did not copurify other proteins (Klose, R.J., 2007; Fang, J., 2007). Like Kdm5, these purifications used epitope tags and standard growth conditions. Budding yeast JmjC domain proteins in-general may thus use a regulatory mechanism in which they have

limited stable interactions under normal conditions but then stably interact with proteins under stress conditions. We note also that Rph1 and Jhd1 purify from yeast as oligomers while Kdm5 purifies as a monomer under normal growth conditions (Klose, R.J., 2007; Fang, J., 2007; Liang, G., 2007) (oligomerization is undetermined for the JmjC proteins Gis1 and Ecm5). While the biochemical implications of this are unknown, they would certainly impact the recruitment of interacting proteins. It would not be surprising if Kdm5 also had latent oligomerization properties that are only revealed under non-normal growth conditions.

The state of S. cerevisiae histone lysine demethylation

S. cerevisiae has five JmjC domain-containing proteins and no obvious LDS1 orthologs. To date, only three of them are verified demethylases; Kdm5 demethylates H3 K4 *in vitro* (Seward, D.J., 2007; Liang, G., 2007; Ingvarsdottir, K., 2007) and Jhd1 and Rph1 demethylate H3 K36 *in vitro* (Fang, J., 2007; Klose, R.J., 2007). Gis1 and Ecm5 remain unconfirmed as demethylases, although Gis1 perturbation reportedly affects H3 K36 methyl levels *in vivo* according to mass spectrometry (Tu, S., 2007). No activity however has been reported *in vitro* or *in vivo* for Ecm5. We note that Gis1 and Ecm5 are difficult to purify (our unpublished results) and such limitations must be overcome before enzymatic activity can be confirmed. Since Kdm5 and Ecm5 are in the JARID family which is known for H3 K4-specific activity (Klose, R.J., 2006), Kdm5 and Ecm5 might work together such that Ecm5's *in vivo* activity is masked by redundant Kdm5 functions. It thus would be interesting to see what phenotypes resulted from Ecm5 and Kdm5 double deletions.

It is noteworthy that *S. cerevisiae* possess more methylated histone lysines than JmjC proteins. Methylated H3 K4 and K36 are demethylated (Liang, G., 2007; Fang, J., 2007; Klose, R.J., 2007) as previously mentioned. Methylated H3 K79 however has no known demethylase in any organism and is very abundant in budding yeast and thus may not be subject to active demethylation (van Leeuwen, F., 2002). Since this manuscript was published, we have confirmed the existence of H4 K20me1 (Edwards, C.R., 2011) and others have confirmed the existence of methylated H2B K37 and H3 K42 (Gardner, K.E., 2011; Hyland, E.M., 2011). Whether any of the five JmjC proteins target them is unknown. While mammalian H4 K20me1 is targeted by PHF8 (Liu, W., 2010), our *in vivo* data argue that the five budding yeast JmjC proteins might not target this mark (Edwards, C.R., 2011).

How do HKMTs and HKDMs together regulate methyllysine levels?

At least three mechanisms can regulate methyllysine levels: increases by methylation, decreases by demethylation, and decreases by dilution during DNA replication, the first and second of which are highly regulatable. HKMTs and HKDMs can utilize two general strategies to maintain appropriate methyllysine levels. Their localization and activity along chromatin can be unregulated, resulting in basal genome-wide methylation or demethylation. Alternatively, they can be recruited to specific locations during specific conditions.

The three known *S. cerevisiae* HKMTs utilize the latter strategy; Set1, Set2, and Dot1 respectively create H3 K4, H3 K36, and H3 K79 methylation which is enriched at genes, and Set1 and Set2 do so proportionately to transcription levels (Pokholok, D.K., 2005). Since we and others have encountered difficulty observing enrichment of Kdm5,

Rph1, and Jhd1 at checked locations via ChIP, and since these three demethylases have few known interacting proteins, the JmjC proteins may utilize the former strategy. In such a scenario, histone methyllysine patterns are maintained by location and condition-specific HKMT activity and continuous genome-wide turnover by HKDM activity and dilution during DNA replication. Alternatively, since another group reported that Kdm5 loss causes delays in H3 K4me3 demethylation at many active genes upon their repression (Radman-Livaja, M., 2010), similar to our results at *GAL1*, the JmjC proteins may utilize the location-specific strategy. Continued efforts to map these proteins' genome-wide distributions will be needed to answer this question.

The future of lysine demethylase studies

While Kdm5, Jhd1, and Rph1 can demethylate histones, it will be interesting to ask what non-histone proteins they also target. The ability of histone modifiers to modify other proteins is increasingly documented in screens and studies of individual proteins. LSD1 for example was shown to demethylate methylated p53 and an *in vitro* acetyltransferase screen identified 91 targets of the HAT complex NuA4 (Huang, J., 2007; Lin, Y.Y., 2009). The failure to show enrichment of Kdm5, Jhd1, or Rph1 at any checked locations leaves open the possibility that they exert their effects on chromatin indirectly by demethylating non-histone proteins. Since Kdm5 loss results in increased Set1/COMPASS recruitment to *GAL1*, whether this complex is demethylated (or methylated) is an obvious starting point.

Unbiased approaches will be advantageous. While the previously mentioned *S. cerevisiae* proteome microarray is an excellent tool for studying the addition of small molecules, it has less applicability for studying the removal of small molecules. An

alternative approach could be to identify Kdm5 interactors, check the interactors for methylation *in vivo*, and then check whether Kdm5 reverses those methylation *in vitro*. This method would be advantageous since it would, at the least, identify Kdm5 regulators. Another possibility would be to construct a proteome microarray using proteins purified from yeast that were grown with radioactive S-adenosyl methionine. Kdm5 would then be incubated with the microarray and loss of radioactivity at specific coordinates would indicate loss of methyl groups. The disadvantage of this strategy would be that radioactive microarrays would have to be constructed and methylations might be missed if they are unabundant *in vivo*.

Another innovative possibility would be to construct a peptide library in which mono-, di-, or trimethylated lysines were surrounded by every possible amino acid sequence. The library would be used as the substrate in *in vitro* demethylase reactions. Demethylation loss could be detected by pooling the peptides and performing mass spectrometry to determine which methylated species had decreased abundance. Alternatively, the peptide library could be constructed on an array that was then incubated with the demethylase, after which demethylation would be detected using pan-methyl-specific antibodies. A disadvantage of this method would be that since enzymes interact with target proteins at multiple contact points, the interactions with peptides may not fully represent the interactions that occur *in vivo*. Ultimately however, identification of non-histone demethylase targets will depend on the prior identification of non-histone methyl sites.

Section 3 Histone Lysine Methylation in *Saccharomyces cerevisiae*

S. cerevisiae Histone H4 Lysine 20 is Monomethylated

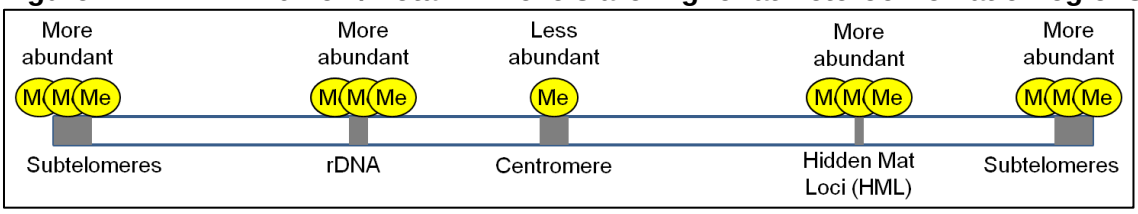
Noting that H4 K20me1 is an interesting but complicated PTM in higher eukaryotes and noting that *S. cerevisiae* lack all the "repressive" histone lysine methylations despite having the corresponding lysines (Martin, C., 2005), we took interest in a mass spectrometry report several years ago suggesting that K20 of budding yeast H4 is monomethylated (Garcia, B.A., 2007). Were this mark present in *S. cerevisiae*, it might be budding yeast's first repressive histone methyllysine and could serve as a model for studying higher eukaryote H4 K20me1. To confirm *S. cerevisiae* H4 K20me1 we performed western analyses with a commercially-available H4 K20me1-specific antibody that showed specificity for the monomethylated state in dot blots of H4 peptides. The antibody detected an epitope in yeast whole-cell extracts that co-migrated with calf thymus H4, had a slower migration if the H4 was FLAG-tagged, was abrogated by an H4 K20R substitution, and was competed by monomethylated but not unmodified H4 peptides. Further, a second commercially-available anti-H4 K20me1 antibody showed similar results and showed no preferential binding of H4 K20me2, 3 peptides in dot blots or peptide competitions of western analyses. These data acquired using two independent antibodies argue strongly that *S. cerevisiae* H4 K20 is monomethylated.

In vivo characterization of H4 K20me1

We performed a variety of assays to characterize H4 K20me1 *in vivo* and determined the following. First, global H4 K20me1 levels do not vary greatly during the cell cycle. Second, H4 K20me1 levels per total H4 levels are most abundant at heterochromatic

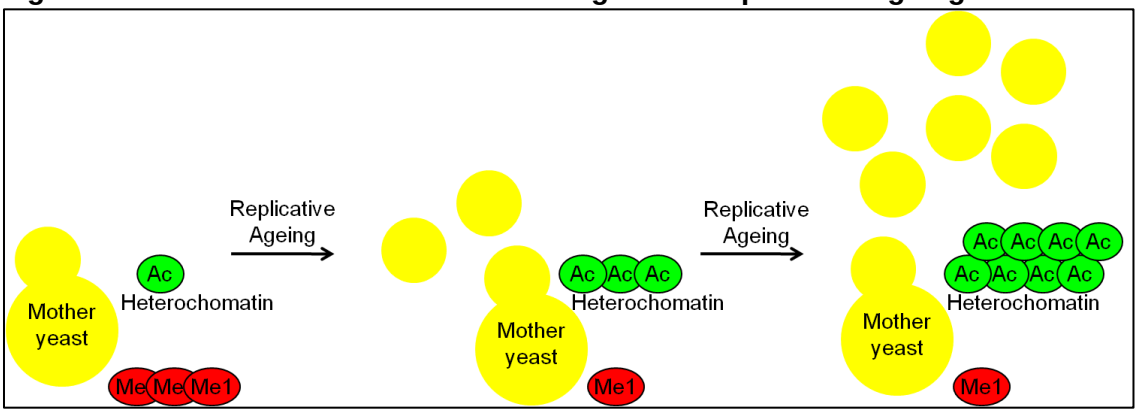
regions (rDNA, subtelomeres, and silent mating type loci), least abundant at centromeres and promoter/5' regions of genes, and have intermediate abundance inside genes. At the genes we checked, H4 K20me1 levels per total H4 levels are slightly higher at the middle regions than the 5' and 3' regions. Third, H4 K20R and K20A substitutions by themselves do not noticeably affect growth with altered nutrient conditions, elevated temperatures, or stresses with DNA replication or damage. Fourth, H4 K20A, but not H4 K20R, causes silencing defects of reporters integrated at heterochromatic regions (rDNA, subtelomeres, and silent mating type loci) and endogenous genes at subtelomere 7L. Importantly, endogenous subtelomere 7L genes had higher H4 K20me1 levels and larger H4 K20A-mediated derepression if they were telomere-proximal than if they were telomere-distal. Fifth, global H4 K20me1 levels decrease during replicative ageing. Sixth, H4 K20R substitutions moderately rescue H3 K56R-mediated Camptothecin-sensitivity, suggesting that H4 K20me1 negatively contributes to survival during DNA damage. (Fig. 1-3)

Figure 1 H4 K20me1 / Total H4 Levels are Higher at Heterochromatic Regions



H4 K20me1 normalized to total H4 is highest at heterochromatic locations including subtelomeres, the silent mating type locus, and rDNA, and lowest at euchromatic locations including centromeres and the promoter/5' regions of some genes.

Figure 2 H4 K20me1 Decreases During Yeast Replicative Ageing

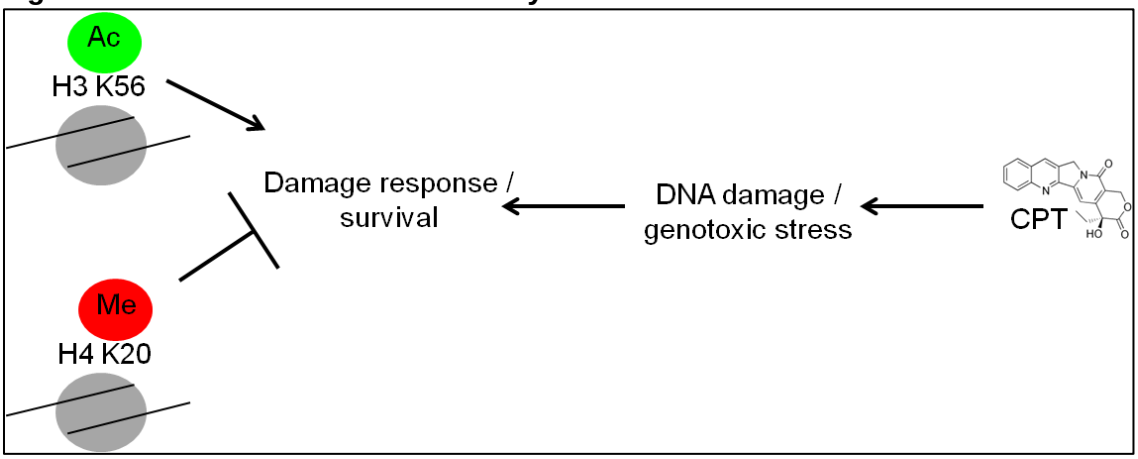


H4 K20me1 normalized to total H4 is globally less abundant in replicatively older than younger yeast, in contrast to H4 K16ac. Green "Ac" ovals are H4 K16ac. Red "Me1" ovals are H4 K20me1. Yellow circles are yeast.

S. cerevisiae H4 K20me1 and Survival During DNA Damage

Noting reports that mammalian H4 K20me1 may recruit DNA damage proteins and may be needed for the DNA damage response (Oda, H., 2010; Oda, H., 2009), we asked whether H4 K20me1 loss in *S. cerevisiae* causes similar defects. While an H4 K20R substitution produced no DNA damage sensitivity, combination with an H3 K56R, but not H3 K79R, substitution resulted in a synthetic effect with Camptothecin, but not vehicle-only, treatment. Unexpectedly, the K20R caused a moderate (~5-fold) rescue of the K56R-mediated sensitivity. Camptothecin (CPT) interferes with topoisomerases such that double-stranded DNA breaks are produced during DNA replication (Li, T.K., 2001). H4 K20R thus moderately rescues the H3 K56R-mediated survival during DNA damage (Figure 3). Rtt109 acetylates newly synthesized H3 K56 to promote recovery from DNA damage during S phase (Masumoto, H., 2005; Han, J., 2007). How H3 K56ac does this is not clear, but it can "loosen" chromatin and promotes nucleosome reassembly at sites of DNA damage after repair. Noting this, several models to explain the K20R rescue can be proposed.

Figure 3 H3 K56R and H4 K20R synthetic effects with CPT



Camptothecin (CPT) compromises topoisomerases, promoting double-stranded DNA breaks during DNA replication. This activates the DNA damage response during which survival is promoted by H3 K56ac and inhibited by H4 K20me1.

First, DNA damage response efficiency is thought to depend on the level of chromatin compaction, with increased compaction being associated with decreased DNA repair efficiency. Presumably, tighter chromatin inhibits access to the DNA by the repair machinery (Lukas, J., 2011). Likewise, acetylated H3 K56, which promotes survival during DNA damage, is thought to "loosen" chromatin by abrogating hydrogen bonding that would normally occur between the DNA backbone and the epsilon nitrogen of lysine 56's side chain (Masumoto, H., 2005). If H4 K20me1 promotes chromatin compaction in *S. cerevisiae*, similar to in higher eukaryotes, and H3 K56ac promotes chromatin decompaction, then loss of H3 K56ac would leave chromatin more compacted and less accessible to the DNA repair machinery whereas simultaneous loss of H4 K20me1 would decrease the compaction and thus slightly uninhibit the repair process. That H4 K20R alone did not promote survival during DNA damage implies that DNA repair and cell growth during Camptothecin treatment are already proceeding at the maximum rate when H3 K56ac is intact.

Second, H3 K56ac is proposed to promote nucleosome reassembly at a DNA damage site after repair completes (Chen, C.C., 2008), presumably by facilitating association with histone chaperones. H3 K56ac-deficient yeast have decreased nucleosome redeposition after repair and this is thought to prevent release from the checkpoint, resulting in decreased growth (Chen, C.C., 2008). If H4 K20me1 inhibits histone deposition, H4 K20me1 loss might compensate for H3 K56ac loss by moderately uninhibiting nucleosome incorporation. Alternatively, since H4 K20me1 may function in mammals by recruiting effectors like 53BP1 (Oda, H., 2010), it may recruit *S. cerevisiae* effectors as well. If it recruits effectors that promote the DNA damage checkpoint, then H4 K20me1 loss might compensate for H3 K56ac loss by inappropriately releasing cells from the damage-induced checkpoint. H4 K20me1 thus might participate in the DNA damage response by affecting chromatin or recruiting PTM readers.

We note that in our studies, H4 K20me1 was detectable at many places throughout the genome and was not enriched during S phase, curious for a mark that may play a role during replication at specific sites of DNA damage. H3 K56ac however is also considered to be spread out along the genome during S phase and it is posited that this allows it to participate in DNA repair without the cell having to target it to specific locations (Masumoto, H., 2005). A similar reason may hold true for H4 K20me1's distribution throughout the genome, and if it functions to balance out H3 K56ac's chromatin "loosening" effects, then H3 K56ac's ubiquitousness may be a reason for H4 K20me1's presence across much of the genome.

S. cerevisiae H4 K20me1 as a Potentially "Repressive" Histone Methyllysine

"Repressive" histone PTMs can have properties including association with transcriptionally repressed genes or compacted regions of the genome, the ability to recruit reader proteins associated with repression or compaction, or the ability to directly promote transcriptional repression of chromatin compaction. The idea that H4 K20me1 is a "repressive" histone PTM is consistent with our results.

First, our ChIP results indicate that H4 K20me1 normalized to total H4 is higher at all known types of *S. cerevisiae* heterochromatin (subtelomeres, rDNA, and the silent mating type locus) than all checked non-heterochromatin locations. Second, our ChIP and gene expression results show a correlation between the locations with highest H4 K20me1 levels and the genes with greatest derepression upon H4 K20A substitution. Third, since acetylation of H3 K56 "loosens" nucleosome structure by disrupting an H3-DNA contact (Masumoto, H., 2005), and since loss of H3 K56ac and H4 K20me1 may have opposing contributions to survival during Camptothecin-mediated DNA damage, it may be that the chromatin decompaction by H3 K56ac, which might promote repair of or survival during the DNA damage response, is moderately counteracted by a compaction effect by H4 K20me1. Fourth, H4 K20me1 decreases globally during yeast replicative ageing whereas H4 K16ac, which is associated with transcriptional activity and open chromatin states, increases globally and at specific subtelomeric locations (Dang, W., 2009). It is thought that increased H4 K16ac at silent subtelomeres causes defective chromatin compaction and silencing. Since H4 K20me1, in contrast, has higher abundance at heterochromatin including subtelomeres and may promote silencing at some subtelomeric genes, and since it has dynamic abundance during ageing that anti-correlates with H4 K16ac, H4 K20me1 may be part of a repressive chromatin state that

is disrupted at heterochromatin by H4 K16ac during replicative ageing. While an H4 K20R substitution did not reproducibly affect replicative lifespan, an H4 K20A substitution has not been checked and whether H4 K20me1's decrease during ageing occurs at heterochromatin has not been checked by ChIP.

While additional work must be done to investigate H4 K20me1's role in the DNA damage response and yeast replicative ageing, there are several particular experiments that would greatly contribute to determining whether it is a "repressive" PTM. First, work must be done to ask whether it recruits compaction or repression-associated proteins (discussed below). Second, experiments asking whether it *in vitro* affects compaction of reconstituted chromatin would be of great value. H4 K20me2, 3 reportedly promote *in vitro* compaction of reconstituted chromatin with *Xenopus laevis* histones (Lu, X., 2008) but H4 K20me1 was not reported and whether it has any such effect on *S. cerevisiae* chromatin is unknown.

H4 K20me1 Conservation from S. cerevisiae to Higher Eukaryotes

While H4 K20me1 is conserved between *S. cerevisiae* and higher eukaryotes, our studies indicate that not all of its properties are conserved. Below is a comparison of its properties in *S. cerevisiae* and higher eukaryotes in terms of genomic localization, responsible HMT, and connections to gene expression, chromatin compaction, effector protein recruitment (or blocking), replicative ageing, the DNA damage response, and DNA replication and the cell cycle.

First, mammalian H4 K20me1 may be associated with silenced regions and indeed is enriched at the inactive X Barr body while Set8 reportedly copurifies with heterochromatin (Wutz A., 2011; Wu, S., 2010). Similarly, budding yeast H4 K20me1

was more abundant at all heterochromatin types than all checked euchromatin regions. Second, mammalian H4 K20me1 exists within genes and tends to be most enriched at 5' or middle regions relative to upstream and downstream regions (Congdon, L.M., 2010; Vakoc, C.R., 2006), similar to *S. cerevisiae* H4 K20me1. Third, mammalian H4 K20me1 promotes or participates in silencing and examples include plasmid reporters, endogenous genes, DNA replication-related genes, and the inactive X Barr body (Congdon, L.M., 2010; Kalakonda, N., 2008; Abbas, T., 2010; Wutz A., 2011). Likewise, budding yeast H4 K20me1 abundance at heterochromatic locations correlates with H4 K20A-mediated derepression of genomic reporters and endogenous genes.

Fourth, mammalian H4 K20me1 promotes chromatin compaction *in vitro* and recruits compaction proteins like L3MBTL1 *in vitro* and *in vivo* (Trojer, P., 2007; Kalakonda, N., 2008). Our studies however have not investigated H4 K20me1's readers or its *in vitro* effects on chromatin structure. Whether this function is conserved is an open question. Fifth, H4 K20me1 levels per total H4 levels globally decrease during *S. cerevisiae* replicative ageing. A connection to replicative potential in higher eukaryotes however is not well-known so this may be a budding yeast-specific phenomenon or may be conserved but unknown in higher eukaryotes. Sixth, mammalian H4 K20me1 may participate in DNA repair by promoting 53BP1 recruitment (Oda, H., 2010; Botuyan, M.V., 2006). Further, H4 K20 methylation loss in *S. pombe* results in DNA damage-sensitivity (Sanders, S.L., 2004). Similarly, *S. cerevisiae* H4 K20me1 may influence cell survival during DNA damage, although the contribution may be negative.

Seventh, mammalian H4 K20me1 is linked to DNA replication and origin licensing and has a characteristic abundance pattern across the cell cycle (Huen, M.S., 2008; Jørgensen, S., 2007; Tardat, M., 2007; Oda, H., 2010; Wu, S., 2010; Abbas, T.,

2010; Pesavento, J.J., 2008; Brustel, J., 2011), whereas our studies of budding yeast H4 K20me1 did not investigate origin licensing and demonstrated neither a link to DNA replication nor a cell cycle-dependent global abundance. Mammalian H4 K20me1's participation in DNA replication thus may not be conserved in *S. cerevisiae* and its cell cycle abundance is definitely not conserved. We note that Hs Set8's cell cycle regulation is partly produced by APC/C-mediated ubiquitylation of its D-box motif during late M phase to trigger degradation (Wu, S., 2010). This D-box motif is conserved in *Homo sapiens*, *Mus musculus*, and *Xenopus laevis* Set8, but not in *Danio rerio*, *Drosophila melanogaster*, and *Caenorhabditis elegans* Set8 (Brustel, J., 2011). H4 K20me1's functions that require its downregulation during parts of the mammalian cell cycle thus may be absent in lower eukaryotes and it would be unsurprising if *S. cerevisiae* H4 K20me1 lacked such cell cycle-related roles.

Eighth, whether mammals and budding yeast are conserved in terms of the responsible HMT is unclear. Mammals (Fang, J., 2002; Nishioka, K., 2002) possess a dedicated H4 K20 monomethyltransferase whereas *S. pombe* (Sanders, S.L., 2004) and *Toxoplasma gondii* (Sautel, C.F., 2007) may possess an HMT that mediates all three methylation states, arguing that K20me1 is more functionally distinct from K20me2, 3 in higher than lower eukaryotes. It is intriguing then that *S. cerevisiae* seem to only possess the monomethylated state of H4 K20. While budding yeast would be expected to utilize a multi-methylation state-creating enzyme like other lower eukaryotes, it likely instead utilizes a dedicated monomethylase like higher eukaryotes.

H4 K20me1 & the Nature of Lower Eukaryote Chromatin

It should be noted that since *S. cerevisiae* was long thought to lack methylation of H3 K9, H3 K27, and H4 K20, all of which are considered "repressive" methylations, *S. cerevisiae* was considered to have chromatin that is more "open" or decompacted than in higher eukaryotes. Like *S. cerevisiae*, *Toxoplasma gondii* was also thought to possess the "open" chromatin lysine methylations (H3 K4, H3 K36, and H3 K79) but not the "repressive" ones and thus was considered to have "open chromatin" similar to budding yeast. It was reported a few years ago however that *T. gondii* chromatin has H4 K20me1-3 created by Tg Set8 (Sautel, C.F., 2007). That H4 K20 methylation exists in *T. gondii* and *S. cerevisiae* argues that the view of the general decompaction of these lower eukaryote genomes based on their lack of "repressive" methylations may not be entirely accurate. We note that a mass spectrometry report detected low levels of H3 K9 methylation in *S. cerevisiae* (Garcia, B.A., 2007) and budding yeast Rph1 can demethylate H3 K9 *in vitro* (Klose, R.J., 2007), suggesting that methylated H3 K9 may exist here. Considering these findings, it may thus be that these lower eukaryotes possess silencing pathways that are simply underappreciated.

The future of S. cerevisiae H4 K20me1 studies

The future of H4 K20me1 studies in *S. cerevisiae* should be focused on determining its phenotypes, methyltransferase, and readers. Considering the number of molecular and biological functions to which mammalian H4 K20me1 is linked, it is likely that *S. cerevisiae* H4 K20me1 is linked to many processes as well. H4 K20 substitutions however have revealed a very limited number of roles in standard phenotypic analyses, perhaps due to redundant mechanisms or the phenotypes being too subtle.

Combinatorial mutations combined with careful analyses may identify more *in vivo* roles and our discovery of a synthetic effect with a topoisomerase poison demonstrates the usefulness of this approach. Our decision to combine an H4 K20R mutation with an H3 K56R mutation during Camptothecin treatment was fortuitous, but it will be more efficient to utilize unbiased approaches like a synthetic genetic array, which would combine H4 K20R with every possible gene deletion. This should however also involve combinations with substitutions of modified histone residues or a histone alanine scan. This is warranted since crosstalk between histone PTMs is well documented but importantly, since proteins interacting with histones likely do so through multiple contact points.

To identify the responsible methyltransferase, our candidate gene overexpression approach could be repeated with epitope-tagged versions of the proteins to verify that the candidate enzymes are expressed. The recent identification however of two other *S. cerevisiae* histone methyllysines with unidentified methyltransferases suggests that the enzyme may not be on our or the other authors' candidate list (Gardner, K.E., 2011; Hyland, E.M., 2011). An unbiased approach such as a genome-wide deletion or overexpression screen will be useful. The overexpression strategy will be more difficult since some proteins might not overexpress well but redundancy and essential genes will not be problematic like they will be for the deletion strategy. Alternatively, an unbiased biochemical approach could be employed in which *S. cerevisiae* whole-cell or nuclear fractions would be tested for *in vitro* H4 K20 methyltransferase activity on histones or nucleosomes.

To identify proteins that bind to or are blocked by this PTM, several options exist. We purified epitope-tagged wild-type or K20R H4 from *S. cerevisiae* and observed copurified proteins with Colloidal Blue and silver protein stains, but too many proteins

were present to notice anything interesting (our unpublished results). Alternatively, wild-type and H4 K20R yeast could be grown with light or heavy amino acid supplements after which material that copurified with H4 could be analyzed by mass spectrometry (SILAC), a method that could better make sense of the purified material. Alternatively, *in vitro* approaches could be used. For example, wild-type or H4 K20me1 peptides immobilized on beads could be incubated with yeast extracts to determine what proteins bind to or are blocked by H4 K20me1. We attempted this but our work was not extensive. An interesting approach could utilize protein microarray technology which has recently been successfully used to identify *in vitro* targets of a HAT (Lin, Y.Y., 2009). Radioactive, epitope, or fluorescently labeled H4 K20me1 peptides could be incubated with an array that contains all known *S. cerevisiae* proteins, only chromatin proteins, or only PTM readers, after which interactions would be followed-up by standard *in vitro* binding assays and *in vivo* coimmunoprecipitation experiments.

Several proteins interact with the patch of basic residues that occupies this part of H4, including the Sir2/3/4 complex, Isw2, and Dot1 (Fazzio, T.G., 2005; Altaf, M., 2007; Fingerman, I.M., 2007), and they might perhaps be blocked by this PTM. However, H4 K16ac and H3 K79me3 levels, which are respectively regulated by Sir2/3/4 and Dot1, are not globally affected by an H4 K20R substitution (our results). Further, H4 K20 substitutions are not known to affect binding or activity of these three proteins and monomethylation may not have any blocking effect on them. A possible binder however might be Rad9, which participates in the DNA damage response. Mammalian 53BP1 and its *S. pombe* ortholog Crb2 bind methylated H4 K20 (binds K20me2 and likely K20me1 to some extent also) as part of the DNA damage response. While they have no overall ortholog in *S. cerevisiae*, budding yeast Rad9 has a tandem tudor domain that is

similar to their tandem tudor domains. The budding yeast Rad9 tandem tudor domain has structural differences that prevent H4 K20me2 binding *in vitro* but whether it binds H4 K20me1 is unreported (Lancelot, N., 2007).

Section 4 NuA4 Acetylation of Spt16 in *Saccharomyces cerevisiae*

NuA4 Acetylates Spt16 In Vitro and Acetylation May Confers Heat-Tolerance In Vivo

A budding yeast proteome microarray recently identified numerous *in vitro* acetyation targets of the NuA4 HAT complex (Esa1 is the HAT) (Lin, Y.Y., 2009). We chose to follow-up the hit Spt16 since it is an important chromatin protein. Spt16 heterodimerizes with Pob3 to form yFACT and it and this complex are conserved in mammals. Although also connected to DNA replication and repair, yFACT's most well-known role is in transcription elongation where it modulates nucleosomes in-front of and behind elongating RNApol2 to respectively allow polymerase passage and maintain sufficient chromatin compaction (Winkler, D.D., 2011). We note that other PTMs reportedly occur on Spt16 and coordinate its various functions (Han, J., 2010). Acetylation may serve a similar regulatory role.

Mass spectrometry of *in vivo* overexpressed Spt16 identified three sites (K493, 583, and 607) but confirmation on *in vivo* Spt16 under normal expression conditions has been unsuccessful using pan-acetyl antibodies both in western analyses and IPs. Interestingly, K→R substitutions (mimics unacetylation) cause moderately decreased heat tolerance at 39 ^{o}C whereas K→Q substitutions (acetyllysine mimics) do not, arguing that this heat-sensitivity results from loss of acetylation rather than loss of the residues or other modifications. How these K→R substitutions cause heat-sensitivity is unknown. They do not impair suppression of cryptic intragenic transcription initiation at checked genes or upregulation of checked heat-response genes. They may cause defects in other yFACT pathways, or they may impair yFACT integrity or Spt16 stability resulting in general loss-of-functions.

Unanswered and new questions about Spt16 acetylation

Many additional questions exist regarding Spt16 acetylation. It is unknown whether any deacetylases remove these marks. If they do, their disruption might increase endogenous acetylation abundance to more detectable levels. It is unknown whether Spt16 is acetylated before or after heterodimerizing with Pob3, in the nucleoplasm or on chromatin, or at some or all of yFACT's target genes. The answers to these questions might reveal roles in yFACT's biogenesis, nuclear localization, and genome-wide recruitment. We note that yFACT's genome-wide localization is not well-studied and examinations of the genome-wide distribution of acetylated Spt16 may have to be paired with this. It is not yet confirmed that NuA4 is the responsible HAT *in vivo* or whether it is the only responsible HAT. Budding yeast acetyltransferases and deacetylases are known for redundancy at histone lysines (Millar, C.B., 2006) and it would be unsurprising if additional enzymes targeted Spt16. Ubiquitylation of budding yeast Spt16 may participate in its DNA replication activities although the residues are unknown (Han, J., 2010). If crosstalk exists between Spt16 ubiquitylation and acetylation, they may work together to coordinate yFACT's different important functions.

The future of acetylation studies

Our work contributes to the growing body of evidence arguing that acetylation is a functional modification not confined to histones but rather widely used by the cell. While acetylation lacks the intrinsic complexity of other modifications like methylation, which can exist in three states on each lysine, its tendency to act synergistically amongst many lysines allows the possibility of a gradient of functionality (Yang, X.J., 2008) or alternatively, redundancy that could ensure continued functionality if some acetylations

are lost. Considering acetylation's utility and how widely used other PTMs like phosphorylation and ubiquitylation are, it should be no surprise that many possible targets exist for NuA4 (Lin, Y.Y., 2009).

Since histone studies have made the importance of acetyllysines clear, and since so many remain to be confirmed and characterized on non-histone proteins, the future of the acetylation field will likely be focused on these non-histone protein acetylations. The number of *in vitro* NuA4 targets and the difficulty that can be encountered when studying each one ensures that much work will remain for some time. We note however that this *in vitro* acetylation screen is only the beginning. This method has revealed its applicability and other *S. cerevisiae* HATs, and HATs from other organisms, should next be subjected to similar experiments to illuminate the full repertoire of non-histone protein acetylation.

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